Anticancer Effects of the Phytochemicals from *Schefflera heptaphylla*

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Abstract

Melanoma is the most aggressive form of skin cancer and is notoriously resistant to all current modalities of cancer therapy. Therefore, the development of novel, melanoma-specific agents is highly warranted. Recent studies showed that betulinic acid, a triterpenoid derived from white birch tree, exhibits strong melanoma-specific activities.

Here, we report the first evidence that a lupane-type triterpenoid 3α-hydroxy-lup-20(29)-ene-23,28-dioic acid (HLDA), purified from the ivy tree *Schefflera heptaphylla* (L.) Frodin, inhibited the growth of cancer cells originated from different histotypes *in vitro* after 72 h of treatment. The values of IC_{50} were 23 μg/ml (melanoma A375), 33 μg/ml (hepatocellular carcinoma HepG2), 40 μg/ml (breast carcinoma MCF-7), 86 μg/ml (prostate carcinoma PC-3), and 90 μg/ml (laryngeal carcinoma HEp2). In addition, the HLDA-rich ethyl acetate fraction from *S. heptaphylla* also inhibited the growth of cancer cells, with the IC_{50} values of 46, 52, 76, 100 and 110 μg/ml, respectively. The most responsive cancer cell line, human melanoma A375, was thus chosen for further analysis on the anticancer properties of HLDA and the ethyl acetate fraction.

Both HLDA and the ethyl acetate fraction at their IC_{50} induced apoptosis in the A375 cells as evidenced by the presence of sub-G_1 cells in DNA flow cytometry and

PARP cleavage in Western blot analysis. However, HLDA did not affect the cell cycle progression of the melanoma cells while the ethyl acetate fraction arrested the cells in the S and G_2/M phase.

HLDA and the ethyl acetate fraction were found to deplete the antiapoptotic Bcl-2 family protein expression (HLDA depleted Bcl-X_L while ethyl acetate fraction depleted Bcl-X_L and Bcl-2) and induce caspase-8 and caspase-9 cleavage in the melanoma cells. Furthermore, the HLDA-induced apoptosis was completely inhibited by pretreatment with general caspase inhibitor (Z-VAD-FMK) and partially blocked by caspase-8 inhibitor (Ac-IETD-CHO) or caspase-9 inhibitor (Z-LEHD-FMK). On the other hand, the ethyl acetate fraction-induced apoptosis was partially blocked by general caspase inhibitor, caspase-8 inhibitor or caspase-9 inhibitor. All these results suggest that both HLDA and the ethyl acetate fraction inhibit the growth of melanoma A375 at least by triggering the mitochondria-dependent apoptosis. Moreover, HLDA-induced apoptosis was mainly executed through caspase-dependent pathway while the ethyl acetate fraction-induced apoptosis involved both caspase-dependent and caspase-independent pathways.

The ethyl acetate fraction exhibited anticancer effects *in vivo* in the DMBA/TPA-induced carcinogenesis model and athymic nude mice model. Both models, the mice were topically treated with the ethyl acetate fraction at doses up to

40 mg/ml. In the skin carcinogenesis model, the papilloma incidence was significantly reduced after treatment twice a week for 20 consecutive weeks, with 100% inhibition of papilloma incidence at the dose of 40 mg/ml. Furthermore, in the athymic BALB/c nude mice model, the tumor size of transplanted melanoma A375 regressed after treatment for 10 days, with 73% tumor growth inhibition at the dose of 2 mg/ml when compared with control mice.

In conclusion, the anticancer activities of HLDA and the ethyl acetate fraction from *S. heptaphylla* on the human melanoma A375 cells are both mediated by apoptosis induction, at least partially, through regulation of Bcl-2 family protein expression and activation of caspases. A further in-depth study to define active agents in the ethyl acetate fraction capable of affording the observed antitumor-promoting effect is highly warranted.

提取自鴨腳木 *Schefflera heptaphylla* 的植物化學成份及其抗癌活性研究

摘要

黑色素瘤屬最具侵略性的皮膚癌之一，而且它對現今的癌症治療存在著很高的抗性。由黑色素瘤衍生的細胞大多顯示抗藥，其多重抗藥性往往是治療癌症的主要障礙。三萜類化合物廣泛存在於植物界，是一種由六個異戊二烯的碳骨架所組成的化合物。其例子包括白樺脂酸，它是由白樺樹中提取的三萜類化合物，報告顯示出它能誘發黑色素瘤細胞的凋亡機制。

在此研究中，我們在鴨腳木中提取了一種羽扇豆烷類三萜類化合物—3α-羥基-20(29)-羽扇豆烷-23,28-二酸 (HLDA)，並証明了它能有效地抑制多種源於不同組織的癌細胞生長。經過三天的體外培養，100 μg/ml 的 HLDA 劑量均能抑制被研究的五種癌細胞的增殖，包括黑色素瘤 A375，肝細胞癌 HepG，乳腺癌 MCF-7，前列腺癌 PC-3 和喉癌 HEp2，其能抑制百份之五十的癌細胞增殖的濃度(IC_{50})分別爲 23, 33, 40, 86 和 90 μg/ml。此外，我們同時亦對萃取 HLDA 的乙酸乙酯分層萃取物加以研究，於同樣的實驗設定下，此分層萃取物的 IC_{50} 分別爲 46, 52, 76, 100 和 110 μg/ml。初步的結果顯示，黑色素瘤 A375 細胞對 HLDA 和乙酸乙酯分層萃取物同樣有著較佳的反應。因此，我們進一步研究兩者對黑色素瘤 A375 細胞的生長抑制作用。經過三天共同培養於 IC_{50} 的 HLDA 和乙酸乙酯分層萃取物中後，透過去氧核糖核酸-流式細胞技術和免疫印跡法分析，我們能了解 HLDA 和乙酸乙酯分層萃取物抑制癌細胞增殖的原因。去氧核糖核酸-流

式細胞技術結果顯示出 HLDA 和乙酸乙酯分層萃取物分別提升了 G_0/G_1 期細胞的數目至二十四及十二倍，指明兩者皆能誘導細胞凋亡；另一方面，免疫印跡法亦檢測了凋亡相關的蛋白水平，其多聚（ADP-核糖）聚合酶 (PARP) 的裂解同樣指出 HLDA 和乙酸乙酯分層萃取物皆誘導細胞凋亡。我們觀察到乙酸乙酯分層萃取物誘導細胞的 S 期和 G_2/M 期周期阻滯並沒有出現在培養於 HLDA 的細胞中。

此研究顯示 HLDA 和乙酸乙酯分層萃取物皆引致抗凋亡的 Bcl-2 家族蛋白表達下降 (前者引致 Bcl-X_L 蛋白表達下降，而後者則引致 Bcl-X_L and Bcl-2 蛋白表達下降)，同時亦剪切了在黑色素瘤細胞內的 caspase-8 and caspase-9。於此，我們利用了天冬氨酸特異性半胱氨酸蛋白酶(caspase)抑制劑去研究 HLDA-誘導的凋亡，總 caspase 抑制劑(Z-VAD-FMK)能完全抑制 HLDA-誘導的凋亡，而 caspase-8 (Ac-IETD-CHO)或 caspase-9 (Z-LEHD-FMK)的抑制劑只能抑制部份乙酸乙酯分層萃取物誘導的凋亡。另一方面，總 caspase、caspase-8 或 caspase-9 抑制劑均只能抑制部份乙酸乙酯分層萃取物所誘導的凋亡。以上的實驗結果顯示出 HLDA 透過 caspase 依賴機制，而乙酸乙酯分層萃取物則透過 caspase 依賴和非 caspase 依賴機制，兩者皆激發線粒體層面所引發的凋亡，從而抑制 A375 黑色素瘤的生長。

我們用了兩個小鼠模型去証明乙酸乙酯分層萃取物於動物體內的抗癌效果。首先，我們利用 7，12-二甲基苯蒽/12-0-十四烷酰法佛醋酸酯-13 誘導乳頭狀瘤於

小鼠背部形成，然後測試乙酸乙酯分層萃取物對此瘤的預防效能。另外，我們將 A375 黑色素瘤細胞注射入裸鼠背部的皮膚下，然後測試乙酸乙酯分層萃取物抑制黑色素瘤細胞增生的能力。於前者實驗中，小鼠經過連續二十星期，每星期兩次高至 40 mg/ml 乙酸乙酯分層萃取物治療後，其乳頭狀瘤的出現率明顯降低，40 mg/ml 的濃度治療更能完全預防乳頭狀瘤的出現。與此同時，連續十天乙酸乙酯分層萃取物治療亦能夠成功地將已射入皮下的 A375 細胞體積減少。

總括而言，我們於此研究中發現，由鴨腳木提煉的 HLDA 和乙酸乙酯分層萃取物皆能誘導 A375 黑色素瘤細胞凋亡。兩者都是通過調節 Bcl-2 家族蛋白的表達，繼而激發 caspase 的活化以致細胞的凋亡。而其於動物體內的正面結果更証明 HLDA 和乙酸乙酯分層萃取物有著被發展爲抗癌藥物的潛能，因此值得對 HLDA 及乙酸乙酯分層萃取物進行更深入的研究。

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Apaf-1	Apoptotic protease activating factor-1
BA	Betulinic acid
BCA	Bicinchoninic acid
Bim	Bcl-2 interacting mediator of cell death
BSA	Bovine serum albumin
Caspase	Cysteine aspartyl-specific proteases
Cyt *c*	Cytochrome *c*
DFF	DNA fragmentation factor
DISC	Death-inducing signaling complex
DMBA	7,12-dimethylbenz[α]anthracene
DTIC	Dacarbazine
DTT	Dithiothreitol
FADD	Fas-associated death domain
FasL	Fas ligand
FDA	Food and Drug Administration
HLDA	3α-hydroxylup-20(29)-ene-23,28-dioic acid
HRP	Horseradish peroxidase
MDR	Multidrug resistance
MEKK	Mitogen-activated protein kinase/ERK kinase kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PARP	poly(ADP-ribose)polymerase
PBS	Phosphate-buffered saline
PI	Propidium iodide

SDS-PAGE	Sodium docecyl sulfate-polyacrylamide gel electrophoresis
TCM	Traditional Chinese medicine
TNF	Tumor necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate

Chapter 1

Introduction

1.1 General Introduction

Traditional Chinese Medicine (TCM) is a comprehensive system of medical thought, pathophysiological concepts, and therapeutic interventions that developed more than 4,000 years. It is practised by about one-fifth of the world's population and is becoming increasingly popular in many medical contexts, particularly among patients with cancer (Cao *et al.*, 2003; Niu and Liu, 2003). TCM encompasses a range of modalities including herbal medicine, acupuncture, medical qigong, dietary recommendations, and meditation. Backed by centuries of experience, China's huge pharmacopoeia contains thousands of plant, animal, or mineral origins, and most of which are herbs. Among the components of TCM, herbal or botanical agents possess complex biological activities that could affect many aspects of carcinogenesis (Tang *et al.*, 2003). At least half of Chinese folk remedies have some kinds of scientific basis for their reputed claims, according to a study by National Academy of Sciences on 796 Chinese herbal remedies (Walters, 1993). The Institute for Traditional Medicine (ITM) made an effort to alert practitioners of Chinese medicine in the U.S. to the promising role for Chinese herbs, with updated information provided over the years.

In recent years there has been a trend to integrate TCM with standard Western medicine in attempts to optimize the treatment outcomes, minimize the side effects of surgery, radiotherapy and chemotherapy, increase the immune function, and to improve survival.

Schefflera heptaphylla is a well-known native species in Hong Kong. It is widely used in combination with other herbs for the treatment of common cold in Hong Kong. Moreover, the wood of this plant has been used to treat cancer in Taiwan (Kan, 1975). A variety of compounds have been isolated from *S. heptaphylla.* These include steroids, sesquiterpenes, diterpenes, triterpenes, and sugars (Kitajima and Tanaka, 1989; Sung *et al.*, 1992). Recently, we have identified a lupane-type triterpene 3α-hydroxylup-20(29)-ene-23,28-dioic acid (HLDA) from the leaves of *S. heptaphylla* (Li, 2004). It has a highly similar chemical structure with betulinic acid, a reported triterpene with anticancer activity, particularly on melanoma (Pisha *et al.*, 1995; Zanon *et al.*, 2004). This prompted us to evaluate the antiproliferative activity of HLDA on different cancer cell lines. Since HLDA was derived from the ethyl acetate fraction of *S. heptaphylla*, the antiproliferative activity of this fraction was also studied.

Kerr *et al.* (1972) originally described two forms of cell death which may occur in the absence of pathological manifestations, necrosis and apoptosis. Necrosis is

characterized by high amplitude swelling of the mitochondria, nuclear flocculation, and uncontrolled cell lysis (Cotter *et al.*, 1990). On the other hand, apoptosis is a programmed cell death controlled by a large number of signaling molecules (Walker *et al.*, 1988). Because deregulated proliferation and inhibition of apoptosis lie at the heart of all tumour development, they present two obvious targets for therapeutic intervention in all cancers (Evan and Vousden, 2001). Chemotherapeutic drugs which cause DNA damage generally kill tumor cells by inducing apoptosis (Cotter *et al.*, 1990; Gatti and Zunino, 2005). Therefore, a better understanding on apoptosis induction and exploitation of apoptosis-based therapies for cancer treatment are crucial (Ghobrial *et al.*, 2005). Due to the improvement of understanding of apoptosis mechanisms, novel strategies have emerged for restoring sensitivity of apoptosis in cancer, and clinical testing of several of these approaches is underway (Reed, 2003).

Tissue culture-based *in vitro* studies help to elucidate many of the autonomous changes that result in cancer and help to find out at least some of the possible anticancer mechanisms of potent compounds. However, it is not possible to accurately model the complex process of tumor formation, invasion, and metastasis entirely *in vitro*. Therefore, after promising anticancer agents have been selected from the *in vitro* studies, information like toxicity and dose-response relationship are usually studied in animal models (Zee-Cheng and Cheng, 1988; Frijhoff *et al.*, 2004). Mouse

has been developed as a tumor-bearing animal in the last 40 years and remains the animal of choice for cancer models. Mouse models of cancer are often good approximations of human cancer counterparts as they recapitulate the multistep nature of tumor progression (Van Dyke and Jacks, 2002). The possible drug-related toxicity can also be studied in the mouse models (Voskoglou-Nomikos *et al.*, 2003). As the extraction yield of the ethyl acetate fraction from *S. heptaphylla* is significantly higher than that of HLDA, it was chosen for further anticancer studies *in vivo*.

Cancer preventive effect of the fraction was studied using the chemically induced skin carcinogenesis model. Cancer therapeutic effect of the fraction, on the other hand, was evaluated with the A375 xenograft in athymic nude mice.

1.2 Literature Review

1.2.1 Cancer and melanoma

Cancer is a growing health problem around the world. According to a recent report by the World Health Oraganization, there are more than 10 millions cases of cancer per year worldwide. Melanoma is the leading cause of skin cancer deaths. It results in an estimated 7400 of the 9600 skin cancer deaths per year. (Bosenberg, 2004). Furthermore, treatment of melanoma is relatively difficult because melanoma is notoriously resistant to conventional chemotherapy. The average survival period of patients with treatment failure is only six to ten months (Soengas and Lowe, 2003).

Melanocytes predominantly locate at the junction of epidermis and dermis, but some are found in hair follicles. Melanocytes produce melanin, a pigment responsible for pigmentation of skin, which is important for protecting the deeper layers of the skin from the harmful ultraviolet radiations. However, melanocytes may sporadically or inherited to mutate and become malignant tumor called melanoma (Haass *et al.*, 2005). The causes of melanoma are related to prolonged exposure to ultraviolet radiation, skin color, and family history of melanoma.

Like other types of cancers, melanoma is caused by abnormal and uncontrolled cell division. Development of cancer is divided into three phases: initiation, promotion, and progression. The initiation phase is a rapid and irreversible process

that occurs when a normal cell is exposed to a carcinogen causing unrepairable or misrepaired DNA damage. The promotion phase, on the other hand, is a protracted process that may require several years to be completed. It consists of the expansion of mutated cells to form an actively proliferating, multicellular premalignant lesion. In the progression phase, another irreversible process occurs over a relatively short period, by which clones with increased proliferative capacity, invasiveness, and metastatic potential are produced (Sun *et al.*, 2004). In this stage, the melanoma metastasizes through the blood stream or lymphatic system to other parts of the body.

1.2.2 Anticancer drugs from natural products

Nature has provided many effective anticancer agents in current use. There are at least 250,000 species of plants, of which more than one thousand plants have been found to possess significant anticancer properties. Various active compounds derived from medicinal plants have been assessed for their efficacy and tolerability in the treatment of cancers. These include paclitaxel and docetaxel from *Taxus brevifolia*; etoposide from *Podophyllum peltatum*; camptothecin from *Camptotheca acuminate*; and vinblastine and vincristine from *Vinca rosea*. They have well recognized anticancer activity in clinics (Mantle *et al.*, 2000). The search for novel anticancer agents from natural sources continues through collaboration among scientists

worldwide (Cragg and Newman, 1999).

Vinblastine and vincristine are two of the alkaloids from *Vinca.* They are used extensively in the treatment of childhood acute lymphoblastic leukemia (Kavallaris *et al.*, 2001), Hodgkin's disease (Rueda Dominguez *et al.*, 2004), testicular cancer (Einhorn, 2002), and breast cancer (Crown, 1998). The clinical interest of *Vinca* alkaloids was clearly identified as early as 1965 and so, this class of compounds has been used as anticancer agents for more than 30 years. They prevent mitosis in metaphase by binding to the beta-tubulin subunit of the alpha/beta-tubulin heterodimer and inhibit polymerization of microtubules (Duflos *et al.*, 2002; Hayot *et al.*, 2002). Unfortunately, despite their usefulness, drug resistance remains a serious clinical problem.

Camptothecin is a monoterpenoid indole alkaloid originally isolated from *Camptotheca acuminata* Decne, a native tree in south China, that has gained great attention for its significant antitumor activities in preclinical studies. Camptothecin and its derivatives are naturally-occurring DNA topoisomerase I inhibitors. Topoisomerase is an important enzyme in DNA replication, which is responsible for the unwinding of the supercoiled DNA. Inhibiting the activity of this enzyme will ultimately lead to cell death. Camptothecin and its derivatives exhibited remarkable anticancer activities and are in clinical trial in treating colon, breast and small cell

lung cancer. Irinotecan (Abigeres *et al.*, 1995; Bleiberg, 1999) and topotecan (Romanelli *et al.*, 1998; Clements *et al.*, 1999), two water-soluble derivatives of camptothecin, have been approved by the Food and Drug Administration (FDA) of the United States of America for treating colorectal and ovarian cancer.

Taxol™ (Paclitaxel) is a taxane alkaloid which has been developed as an anticancer drug in the past three decades. In 1971, taxol was isolated and identified from the stem bark of the western yew, *Taxus brevifolia*, which is found in the northwest Pacific coastal region of the United States (Wani *et al.*, 1971). In 1992, taxol was approved for treatment of refractory ovarian cancer and today is used against breast and non-small cell lung cancers and Kaposi's sarcoma (Oberlies and Kroll, 2004). It was approved by the FDA for first-line therapy for the treatment of advanced carcinoma of the ovary in combination with cisplatin in 1998. It binds to the N-terminal region of beta-tubulin and promotes the formation of highly stable microtubules that resist depolymerization, thus preventing cell division and arresting the cell cycle at the G_2/M phase (Schiff and Horwitz, 1980; Wang *et al.*, 2000). Unfortunately, taxol is subjected to the problem of multidrug resistance of metastatic melanoma. In early trials with treatment naïve previously untreated metastatic melanoma patients, it produced a reported response rate of 16% when it was given as a 24 h infusion (Nathan *et al.*, 2000).

1.2.3 Challenges in treatment of melanoma

Treatment options of melanoma are limited because of the poor response of melanoma to current therapies. Treatment with the alkylating agent dacarbazine (DTIC), the only single-agent approved by the FDA for treating metastatic melanoma, allows a complete response rate of only 16% (Serrone *et al.*, 2000). Other chemotherapeutic agents including vineristine, vinblastine, cisplatin, paclitaxel, docetaxel, and the DTIC analogue temozolomide were also failed in large randomized studies (Soengas and Lowe, 2003). Based on their single-agent activities, several combination chemotherapies have been investigated with preliminary results appeared promising. However, in randomized phase III trials the two most active chemotherapy combination regimens, cisplatin combined with vinblastine, and DTIC combined with the Dartmouth regimen (DTIC, cisplatin, bischloroethylnitrosourea, and tamoxifen), did not prove to be superior to single-agent DTIC for overall survival (Atallah and Flaherty, 2005).

Melanoma-derived cell lines are often markedly chemoresistant, suggesting that some cellular mechanisms could mediate the generation of multidrug resistance (MDR) phenotype. This phenotype is often due to the expression of P-glycoprotein and the MDR-related proteins (Schadendorf *et al.*, 1995), which are drug transporters associated with the resistance to a broad spectrum of lipophilic drugs (Ichihashi and

Kitajima, 2001). MDR can be resulted from several distinct mechanisms, including alterations of tumor cell cycle checkpoints, impairment of tumor apoptotic pathways, repair of damaged cellular targets, and reduced drug accumulation in tumor cells. (Gottesman *et al.*, 2002). Taxol and vindesine are common anticancer drugs being used, but both drugs are subjected to the problem of MDR in melanoma (Grottke *et al.*, 2000; Nathan *et al.*, 2000). It is noteworthy that MDR is a common phenomenon in malignant melanoma and therefore provides motivation for scientists to explore alternative approaches and to search for new anticancer agents for treatment of melanoma.

1.2.4 TCM – New source of natural products for cancer therapy

Traditional Chinese medicine (TCM) is a hot issue in cancer therapy nowadays. It is an alternative method of cancer therapy, which can be administered in oral, topical, or injectable forms. Among the components of TCM, herbal or botanical agents possess complex biological activities that could affect many aspects of carcinogenesis such as cell growth and proliferation, apoptosis, host-tumor interactions, immune function, and differentiation (Koo and Desai, 2003).

For more than 30 years, Chinese herbs and materials derived from the herbs have been used as adjunct therapies for cancer patients. The application of TCM was first

developed clinically in China and Japan during the 1970s and was relayed to the rest of the world in 1983 through an international conference in Beijing which was followed up by press reports in English and other languages. Many anticancer drugs originated from plants in China have been investigated. The continuous search for new anticancer drugs from plants will be a fruitful frontier in cancer treatment and chemoprevention.

The basic concepts of drug therapy in traditional Chinese medicine and modern Western medicine are very different (Cheng, 2000). In China, many drugs isolated from plants have been used as herbal medication before the active principles were identified. Some theories such as the yin-yang theory and the five-element theory were commonly employed to explain the rationale of using TCM. Drug therapy has been one of the means used in Chinese medicine to keep these elements and the flow of energy in balance (Borchardt, 2003). However, for the modernization of TCM, active components in TCM for treating the disease and the underlying mechanisms have to be identified, as in the modern Western medicine.

1.2.6 The genus *Schefflera*

The whole *Schefflera* genus comprises of approximately 200 species which distribute in the tropical and subtropical areas of the world. Thirty seven species of

them are found in the southern China (Chen *et al.*, 2002). In the "Zhong Hua Ben Cao", some of them are recorded as medical plants, including *S. arboricola* (七葉蓮), *S. bodinieri* (川黔鴨腳木), *S. delavayi* (大泡通), *S. kwangsiensis* (漢桃葉) and *S. venulosa* (密脈鵝掌柴). Plants of the *Schefflera* genus are clinically used by Chinese herbal practitioners for the treatment of pain, fever, headache, rheumatic arthritis, bone fracture, sprains, pharyngitis, dermatitis, eczema and lumbago in Asian countries (Li, 1999). Many of them have been reported for their antinociceptory (Zhu *et al.*, 1996), anti-inflammatory (Shen *et al.*, 1998), antiviral (Li *et al.*, 2004), anti-malarial (Tetyana *et al.*, 2002), and antifungal activities (Muir *et al.*, 1982).

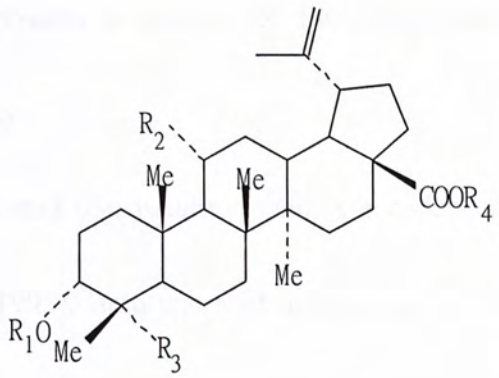
Previous reports indicated that *Schefflera* plants exhibit anticancer activities. Kuo and colleagues reported that the ethyl acetate fraction of *S. taiwaniana* exhibited significant growth inhibitory activity in four human cancer cell lines, hepatoma (59T), colon cancer (DLD1), nasopharyngeal carcinoma (HONE1), and gastric cancer (SCM1) (Kuo *et al.*, 2002). Besides, the saponins isolated from *S. fagueti* showed significant inhibitory effects on three cell lines, hybridoma (J774), human kidney cancer cells (HEK-293), and fibrosarcoma (WEHI-164) (Cioffi *et al.*, 2003; Braca *et al.*, 2004). In particular, betulinic acid (BA) has demonstrated selective cytotoxicity over a number of specific tumor types and its anticancer activity was confirmed in mice bearing human xenografts (Zuco *et al.*, 2002; Eiznhamer and Xu, 2004). This

compound is currently undergoing preclinical development for the treatment or prevention of a number of malignant tumors.

Schefflera octophylla (Lour.) Harms. is one of the best-known native tree species in Hong Kong. It is instantly recognizable by its palmately compound leaves, with 5 to 10 leaflets radiating from the end of the stalk. However, in 1990, David Frodin reported that the tree should actually be called *S. heptaphylla* (L.) Frodin. (Frodin, 1990). It is one of the principal ingredients of the herbal tea 'Twenty-four items tea' (二十四味), which is widely used for the treatment of common cold in Hong Kong. The antiviral activities of *S. heptaphylla* have been reported (Li *et al.*, 2004). However, its anticancer effect has not been investigated.

Triterpenoids are compounds with a carbon skeleton based on 6 isoprene units which are widely found throughout the plant kingdom. They have relatively complex cyclic structures, most being alcohols, aldehydes, or carboxylic acids. Thousands of structures have been reported with hundreds of new derivatives described each year. Triterpenoids and their glycosides, called saponins, are the major chemical constituents of *S. heptaphylla*. These triterpenoids and their glycosides can be divided into different types according to their structures, including lupane-type, ursane-type and oleanane-type (Sung and Adam, 1991*a*; Sung *et al.*, 1991*c*; Sung *et al.*, 1992; Tran and Adam, 1992; Maeda *et al.*, 1994). Three triterpenoids have been isolated

from *S. heptaphylla* (Sung *et al.*, 1991*b*). They are betulinic acid, 3α-hydroxy-lup-20(29)-ene-23,28-dioic acid, and 3α,11-α-dihydroxy-lup-20(29)-ene-23,28-dioic acid. The structures of 3α-hydroxy-lup-20(29)-ene-23,28-dioic acid and betulinic acid are the same except the methyl group of betulinic acid at carbon-23 is replaced by a carboxyl group in 3α-hydroxy-lup-20(29)-ene-23,28-dioic acid (Fig. 1.1). However, the bioactivities of this triterpenoid have not been investigated.



HLDA: $R_1 = H$, $R_2 = H$, $R_3 = COOH$, $R_4 = H$

Molecular formula of $C_{30}H_{46}O_5$

MW: 486.33

BA: $R_1 = H$, $R_2 = H$, $R_3 = CH_3$, $R_4 = H$

Molecular formula of $C_{30}H_{48}O_3$

MW: 456.36

Fig. 1.1 Chemical structures of 3α-hydroxylup-20(29)-ene-23,28-dioic acid (HLDA) and betulinic acid (BA)

1.2.7 Anticancer activities of triterpenoids

Triterpenoids have been tested for a variety of biological activities. It is shown that many triterpenoids possess a number of intriguing pharmacological effects including anticancer activity.

Previous reports indicated that betulinic acid is a melanoma-specific cytotoxic triterpenoid (Pisha *et al.*, 1995). Betulinic acid induced cell cycle arrest at G_1 phase and caused apoptosis in human melanoma (B16F10) cells (Sawada *et al.*, 2004). Other studies showed that betulinic acid triggers apoptosis in human colorectal carcinoma (HCT 116) and prostate carcinoma (DU145) by a direct effect on mitochondria through caspase-3 activation (Fulda *et al.*, 1998).

F035, a triterpenoid saponin, and two pure biologically active derivatives from *Acacia victoriae*, an Australian desert tree of the Leguminosae family, markedly inhibited the growth of several tumor cell lines with minimum growth inhibition in human foreskin fibroblasts, mouse fibroblasts, and immortalized breast epithelial cells. The triterpenoid saponins induce cell cycle (G_1) arrest in human breast cancer (MDA-MB-453) and apoptosis in Jurkat cells (T-cell leukemia) (Mujoo *et al.*, 2001).

1.2.8 Cancer and apoptosis

There are two major mechanisms of cell death, necrosis and apoptosis. Necrosis, frequently initiated by damage to the plasma membrane, represents a passive form of cell death induced mainly by non-physiological agents and is often accompanied by autolysis of the cell. In contrast, apoptosis is a cell suicide mechanism which is important in controlling cell number and proliferation as part of normal development. It is defined by a stereotypical series of biochemical and morphological changes, including cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing, caspase activation, and the formation of membrane bound vesicles termed as apoptotic bodies (Hengartner, 2000). Apoptotic cells are rapidly eliminated by phagocytic cells without eliciting a significant inflammatory damage to surrounding cells (Kerr *et al.*, 1972).

Apoptosis, or programmed cell death, is a key regulator of tissue homeostasis, which critically depends on the balance between cell proliferation and cell death. (Evan and Vousden, 2001). Apoptosis induction is one of the important anticancer therapeutic routes because nearly all anticancer approaches, such as anticancer drugs, γ-irradiation or immunotherapy, act primarily by inducing apoptosis in target cells (Ghobrial *et al.*, 2005; Lockshin and Zakeri, 2004). Thus, apoptotic regulation is a

critical determinant of carcinogenesis and the therapeutic responsiveness of cancers (Konstantinidou *et al.*, 2002; Sun *et al.*, 2004).

This mode of cell death engages well-ordered signaling cascades, in which loss of mitochondrial membrane potential through the action of the Bcl-2 family of proteins, caspase activation, and DNA fragmentation are the hallmarks (Strasser *et al.*, 2000). The apoptotic pathways can be initiated from different entry sites. For example, active signaling can be initiated from death receptors, such as CD95 and TRAIL receptors, or at mitochondria through actions of the Bcl-2 family proteins (Fig. 1.2).

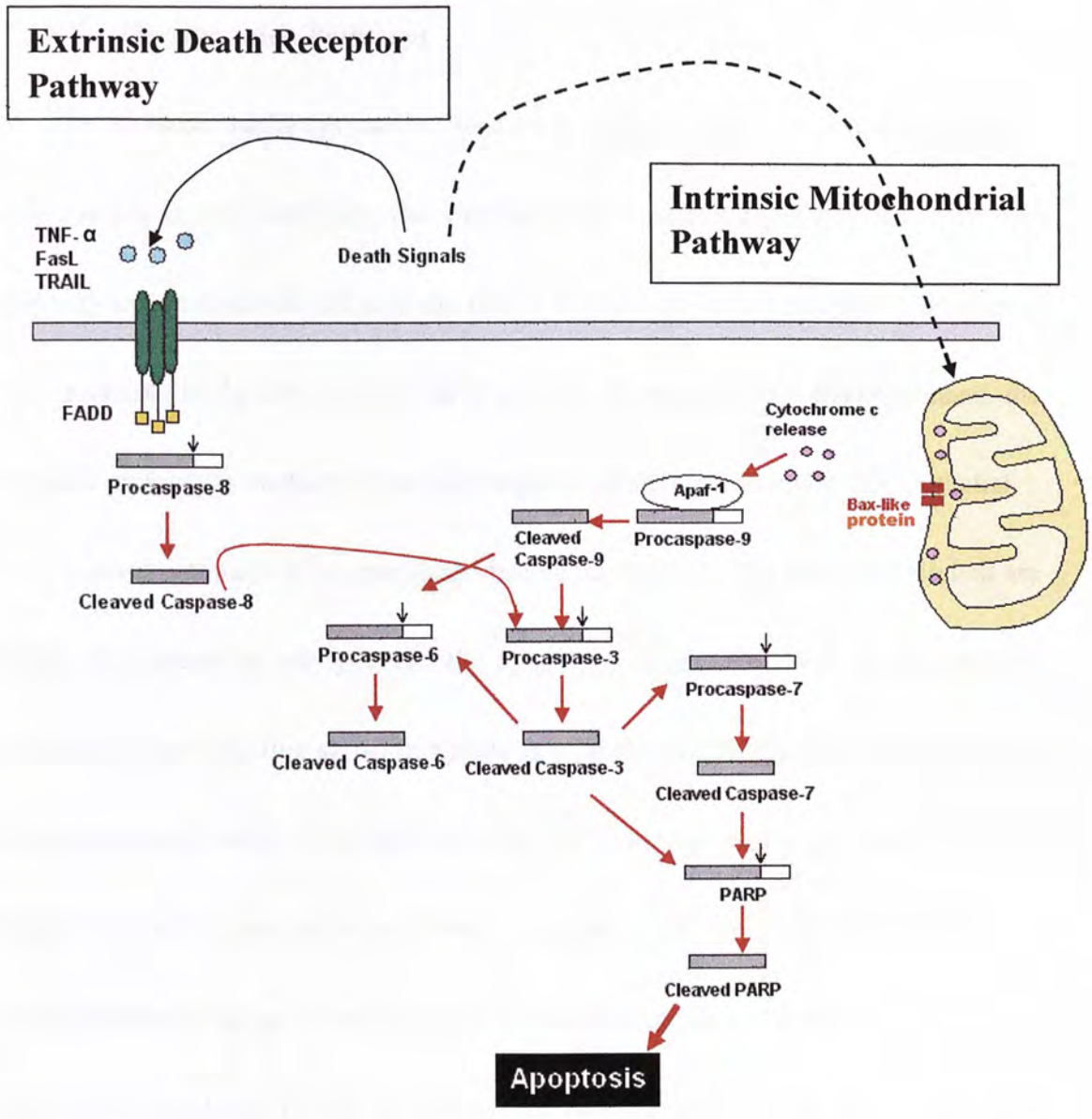


Fig. 1.2 A simplified diagram showing the extrinsic and intrinsic pathways of apoptosis. Apoptosis occurs through two main pathways. The extrinsic pathway is a receptor-linked pathway that is initiated by ligand binding to death receptors on the cell surface. Alternatively, the intrinsic pathway is mediated through changes in mitochondrial membrane potential and release of apoptogenic molecules such as cytochrome *c* from the mitochondria, which is controlled by the Bcl-2 family proteins. (Modified from Philchenkov, 2004 and Wang *et al.*, 2000)

1.2.8.1 The Apoptosis Pathways

The apoptosis pathways can be initiated by different entry sites and assigned to two categories for simplicity, the extrinsic and intrinsic pathways. The extrinsic pathway is a receptor-linked pathway that is initiated by ligand binding to a receptor on the surface of the cell, such as CD95 and TRAIL receptors. On the other hand, the intrinsic pathway is mediated through changes in mitochondrial membrane potential.

Several pathways of apoptosis had been described, and the pathways elicited are highly dependent on cell type and the apoptotic stimuli. Hundreds of proteins are involved in the molecular signal transduction of apoptosis (Hengartner, 2000). Among them, the Bcl-2 family members, cytochrome *c* and apoptosis protease activation factor-1 (Apaf-1) are found extremely important in apoptosis that elicited by chemotherapeutic drugs. Cystein aspartate-specific proteases, also known as caspases, are another important family of intracellular proteins involved in the initiation and execution of apoptosis (Cohen, 1997; Earnshaw *et al.*, 1999).

Caspases

Mechanisms associated with caspase activation in the extrinsic and intrinsic apoptotic pathways have been characterized extensively (Hengartner, 2000; Ghobrial *et al.*, 2005). Both apoptotic cascades are activated in response to a wide variety of

stimuli including stress, cytotoxic drugs, ionising radiation, and withdrawal of survival factors (Zabkiewicz and Clarke, 2004).

Caspases can be subdivided into two general categories: initiator caspases and effector caspases. The effector caspases, including caspases 3, 6 and 7, are those that attack critical cytoplasmic proteins like cytoskeletal proteins, polyadenosylribose polymerase, and other strategic enzymes or structural proteins. The effector caspases typically exist as proenzyme in the cytoplasm and are proteolytically activated by the initiator caspases, including caspases 8, 9 and 10 (Fulda and Debatin, 2004). Usually, initiator caspases, once activated, will activate the downstream effector caspases in a cascade-like pattern (Slee *et al.*, 1999; Thornberry and Lazebnik, 1998). Caspases can activate each other by cleavage at identical sequences, resulting in amplification of caspase activity through a protease cascade (Thornberry and Lazebnik, 1998). In turn, the downstream effector caspases cleave a number of different substrates in the cytoplasm or nucleus, contributing to morphological and biochemical alterations and characteristics of apoptosis. For example, poly(ADP-ribose)polymerase (PARP), mitogen-activated protein kinase/ERK kinase kinase (MEKK), DNA fragmentation factor (DFF), and others (Cohen, 1997; Fulda and Debatin, 2004). Likewise, proteolysis of several cytoskeletal proteins such as actin (Mashima *et al.*, 1997), fodrin (Janicke *et al.*, 1998), and lamin (Orth *et al.*, 1996) may initiate morphological

alterations observed during apoptosis including cell shrinkage, loss of cell-matrix and cell-cell interactions and formation of apoptotic bodies.

PARP is localized to the centrosomes and the chromosomes. It is a nuclear enzyme that is activated by DNA strand breaks. Once activated, it is actively involved in DNA repair. At physiological conditions, PARP plays an important role in maintaining genomic stability, controlling homologous recombination, and regulating centrosome function (Kanai *et al.*, 2000; Tong *et al.*, 2001). PARP activation usually results from caspase-3 activation (Cryns and Yuan, 1998), no matter in animal or plant cells, although some studies reported that PARP cleavage can also be stimulated by caspase-7 (Germain *et al.*, 1999).

Bcl-2 family proteins

The Bcl-2 family proteins are the central regulator of apoptosis. It comprises both antiapoptotic and proapoptotic members. The expression ratio of proapoptotic to antiapoptotic members is critical to the cellular decision to live or die (Herzig and Christofori, 2002).

Antiapoptotic members of the family (e.g. Bcl-2, Bcl-X_L, Boo and Bcl-w) inhibit apoptosis and promote cell growth. On the other hand, the proapoptotic members (e.g. Bax, Bak, Bok) promote apoptosis and drive cell to die (Cory and Adams, 2002). It

has been suggested that the antiapoptoic Bcl-2 family members function, at least in part, by interacting with and antagonizing proapoptotic family members (Opferman and Korsmeyer, 2003). Imbalanced expression among the Bcl-2 family of proteins, in favor of the antiapoptotic members, is a phenomenon frequently occured in cancer cells. Overexpression of Bcl-2, Bcl-X_L or other antiapoptotic homologues occurs in more than half of all cancers (Kabore *et al.*, 2004; Osford *et al.*, 2004). Moreover, loss of proapoptotic Bax and Bak is also a common feature in human tumors (Meijerink *et al.*, 1998).

The proapoptotic Bcl-2 homologues Bax and Bak are inserted in the outer mitochondrial membrane upon their oligomerization, thus forming new channels and enlarging the existing permeability transition pore. This promotes cytochrome *c* release from the mitochondria (Borner, 2003). The antiapoptotic family members like Bcl-2 and Bcl-X_L hold Bax and Bak in check, possibly by forming heterodimers with these molecules, thereby preserving mitochondrial integrity.

The Extrinsic death receptor pathway

The extrinsic pathway is a receptor-linked pathway that is initiated by ligand binding to the death receptors of the tumor necrosis factor (TNF) receptor superfamily, which includes members such as CD95 (APO/Fas) or TRAIL receptors. The death

receptors of the tumor necrosis factor receptor (TNFR) superfamily such as Fas and TRAIL receptors share a distinct domain within their cytoplasmic tails called death domain (Garg and Aggarwal, 2002). When Fas ligand (FasL) binds to and induces clustering of Fas, activation of these receptors results in the recruitment of several intracellular proteins like Fas-associated death domain (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC), and caspase-8 is thus activated (Ashkenazi, 2002).

Upon cleavage of procaspase-8 and subsequent activation, caspase-8 can then initiate the proteolytic activation of caspase-3 and caspase-7 that eventually leads to apoptosis. The extrinsic and intrinsic apoptotic pathways can be interconnected at different levels. Caspase-8 can either directly cleaves and activates the effector caspase-3 in type I cells or cleave the Bid in type II cells (Scaffidi *et al.*, 1999; Barnhart *et al.*, 2003). A mitochondrial amplification loop can be initiated by caspase-8-mediated cleavage of Bid in response to death receptor activation. The truncated Bid (tBid) translocates to the mitochondria and leads to the oligomerization of Bax and Bak that eventually initiated the intrinsic apoptosis pathway (Wei *et al.*, 2000).

Intrinsic mitochondrial pathway

The intrinsic pathway, on the other hand, involves activation of the proteolytic caspases in the cytosol by the apoptogenic molecules released from mitochondria, under the control of the Bcl-2 family proteins (Gross *et al.*, 1999). Under apoptotic stimuli, mitochondria become permeable due to the loss of mitochrondrial membrane potential, which in turn release apoptogenic factors such as cytochrome *c*, apoptosis-inducing factor and/or endonuclease G from the mitochondrial intermembrane space (van Loo *et al.*, 2002). Some of the proapoptotic Bcl-2 proteins, such as Bax, Bak and Bid, normally reside in the cytosol but translocate to mitochondria upon the apoptotic stimuli, where they promote release of the apoptogenic molecules (Antonsson *et al.*, 2000). However, their proapoptotic activities can be antagonized by the prosurvival Bcl-2 family proteins such as, Bcl-2 and Bcl-X_L (Oltvai *et al.*, 1993). The released cytochrome *c* then associates with the apoptotic-protease-activating factor-1 (Apaf-1) and procaspase-9, together with dATP or ATP, to form a structure called apoptosome (Cain *et al.*, 2002). The initiator caspase-9 is thus activated within the apoptosome, which then cleaves and activates effector caspase-3, -6 and -7 (Zhivotovsky, 2003). The activated effector caspases then cleave their corresponding death substrates such as PARP. Cleavage of the target proteins results ultimately in apoptotic cell death (Cohen, 1997). As mentioned before,

the extrinsic and intrinsic apoptotic pathways can be interconnected at different levels, and a mitochondrial amplification loop can be initiated by caspase-8-mediated cleavage of Bid in response to death receptor activation. Truncated Bid (tBid) then induces Bax and Bak to oligomerize (Wei *et al.*, 2000), which in turn triggers the release of apoptogenic factors. The intrinsic pathway is therefore activated.

1.2.9 Studies of anticancer molecules against melanoma

Melanoma is extremely resistant to chemotherapeutic drugs, and the apoptotic indices are typically low in melanoma tumors (Staunton and Gaffney, 1995). Thus, identification of molecules involved in the regulation and execution of apoptosis is important for treating melanoma.

1.2.9.1 *In vitro* models for studying anticancer molecules

Tissue culture-based experiments are important to elucidate many of the autonomous changes in cancers and to study the therapeutic mechanisms of anticancer drugs. Due to the ease of establishment and maintenance, *in vitro* models are attractive especially for screening purposes (Voskoglou-Nomikos *et al.*, 2003).

Screening for anti-cancer substances is commonly conducted using viability assays (Hagg *et al.*, 2002). The present human tumor cell line *in vitro* screen is

technically simple, relatively fast, cheap, reproducible, and provides valuable indicative data of mechanistic activity and target interaction (Suggitt and Bibby, 2005).

Cell-based assays are particularly valuable compared with cell-free assays when searching for therapeutic agents. Because they select not only for activity against a particular target but also for other properties, such as the permeability to cells and the activity of the agent to retain in tissue culture medium and in cells (Roberge *et al.*, 2000). However, the limitations of *in vitro* method do exist. An inherent problem with this approach is that all compounds that are toxic and growth inhibitory, irrespective of mechanism of action, will score positive. It would be beneficial to be able to screen for compounds that specifically induce apoptosis (Hagg *et al.*, 2002). It is also clear that factors other than the inherent chemosensitivity of tumor cells significantly influence the outcome of chemotherapy *in vivo* (Suggitt and Bibby, 2005).

Assays for measuring anticancer activity *in vitro*

Cell density, viability, proliferation status and metabolic activity are the most important parameters to assess the efficacy of drugs in cell-based anticancer research. Several assays have been developed targeted to measure these parameters.

Trypan blue exclusion is the most widely accepted method for enumerating living and dead cells in culture. Trypan blue will penetrate and stain dead cells that have lost membrane integrity. On the contrary, the dye will be excluded by the viable cells with intact cell membranes. Thus, the viability and cell density can be determined simultaneously by direct counting of cells with the aid of hemacytometer.

A relatively simple, indirect, colorimetric method for the quantitative measurement of surviving cells was introduced by Mosmann in 1983, which was named as MTT assay. The assay is based on the capacity of mitochondrial dehydrogenase enzymes of viable cells to transform the yellow, water-soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into its blue, water-insoluble formazan derivative (Mosmann, 1983). A linear relationship was demonstrated between the formazan products and the number of viable cells (Ferrari *et al.*, 1990). Because of its high sensitivity and reproducibility, it was widely used to monitor the sensitivity of human tumor cells to chemotherapeutic agents (Cole, 1986). Since the process of apoptosis is complex and not single feature can distinguish between apoptosis and necrosis, both flow cytometry and western blot techniques were therefore used in our study to confirm the mode cell death induced by HLDA and the ethyl acetate fraction. Flow cytometry has been applied in basic research and in the clinic to identify and measure apoptotic cells. Flow cytometry is

an innovative technology which involves the use of a beam of laser light projected through a liquid stream that contains cells. The signals produced are then converted for computer storage and data analysis, and can provide information about various cellular properties (Culmsee and Nolte, 2002). The apoptotic cells, but not necrotic ones, have reduced DNA stainability following staining with a variety of fluorochromes such as propidium iodide (PI) (Darzynkiewicz *et al.*, 1992). Therefore, the presence of cells with DNA stainability lower than that of G_1 cells (i.e. sub-G_1 peak) in the DNA histogram has widely been considered as a specific marker of cell death by apoptosis (Bertho *et al.*, 2000; Darzynkiewicz *et al.*, 1992; Nicoletti *et al.*, 1991).

Western blotting is a method in molecular biology for detecting and measuring semi-quantitatively the expression of the protein of interest in a sample using specific antibody. In cancer research, this technique has been extensively used for investigating the alterations of protein expressions of cancer cells induced by anticancer agents, giving light on the possible therapeutic routes of the agent. Apoptosis is characterized by internucleosomal DNA fragmentation and cleavage of many housekeeping proteins such as poly(ADP-ribose) polymerase (PARP). PARP involves in DNA repair is important for cells to maintain their viability. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing

apoptosis. It is thought to be one of the earliest targets for cleavage by caspase-3-like proteases during apoptosis (Boulares *et al.*, 2002).

1.2.9.2 *In vivo* models for studying anticancer molecules

Before a particular drug or procedure can be tested on human, antitumor *in vivo* studies have to be shown to be positive in laboratory animals. Mouse has been extensively developed as a tumor-bearing animal in the last 40 years and remains the animal of choice for cancer models. Since mouse and human skin share many characteristics, mouse models of cancer are often good approximations of human cancer counterparts and recapitulate the tumor-stromal interactions, angiogenesis, and multistep nature of tumor progression of cancers (Van Dyke and Jacks, 2002). The response of a tumor to an experimental drug is studied in the context of a living organism with a fully functional metabolic system and the potential to exhibit possible drug-related toxic effects can be studied using *in vivo* model (Voskoglou-Nomikos *et al.*, 2003). Thus, these models will likely aid in the development and testing of novel therapeutic approaches. In the 1950s, spontaneously arised or chemically induced tumors of mice were used. Transplantation of human tumors in mice became possible with the development of technologies for creating immunocompromised mice and

with the discovery of nude mice (Voskoglou-Nomikos *et al.*, 2003). These models aid in the development and testing of novel therapeutic approaches.

Chemically induced skin carcinogenesis model to study cancer preventive effect

Using chemical carcinogens to induce squamous lesions in rodents has been extensively characterized (Yuspa and Poirer, 1988). Most commonly, a two-step process is used with 7,12-dimethylbenz[α]anthracene (DMBA) as the initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as promoter. Typically papillary/polypoid exophytic squamous lesions (papillomas) arise first (Klein-Szanto *et al.*, 1994). Squamous papillomas with a craterlike growth pattern and a polypoid stalk are very common in mice treated with chemical carcinogens.

Chemoprevention is the use of agents to slow down the progression of, reverse, or inhibit carcinogenesis, thereby lowering the risk of developing invasive or clinically significant disease. Because the initiation and progression phases are irreversible and are relatively transient events, the promotion phase of carcinogenesis may provide the best target for cancer prevention (Sun *et al.*, 2004). The occurrence and development of tumors may be prevented and blocked if some effective interference factors are brought into play.

Skin carcinogenesis can be operationally and mechanistically divided into at

least three major stages, initiation, promotion and progression. The multistage model of mouse skin carcinogenesis has been extremely useful for studying various factors that modify the carcinogenic process (Slaga *et al.*, 1996). It is also an ideal system to study the timing of qualitative and quantitative alterations which takes place during the different stages of chemical carcinogenesis, allowing analysis of the events that lead to the transition from the stage of initiation to the stage of promotion and finally to the progression of carcinogenesis (Murakami et al., 2004; Zoumpourlis *et al.*, 2003). Most commonly, a two-step process is used by using DMBA as the initiator and TPA as the promoter (Owens *et al.*, 1999; Bai *et al.*, 2004; Ding *et al.*, 2004). Using the DMBA/TPA model system, one can specifically study the effects of potential modifiers on the initiation and the promotion stages independently (DiGiovanni, 1991). For these advantages, the DMBA/TPA model was chosen in this study to investigate the cancer preventive effects of the ethyl acetate fraction *in vivo*.

Human xenograft model to study cancer therapeutic effect

Congenitally athymic nude mice (nu/nu) are presented as a model for the study of cell-mediated immunologic deficiencies. These mice possess a vestigial thymus which is incapable of producing mature T-cells as shown by a decreased lymphocyte population carrying the theta antigen and depleted 'thymus-dependent' areas in their

peripheral lymphoid organs (Pelleitier and Montplaisir, 1975). They have been used in recent years to study the biology of human tumors and to assess the therapeutic responses *in vivo* because their compromised immune system makes them impossible to reject foreign cells (called xenografts). The first human xenograft was created in 1969 (Rygaard and Povlsen, 1969), in which the xenografts were implanted subcutaneously into the immunosuppressed mice. This model has then played a significant role in preclinical anticancer drug development for the past 25 years (Brady *et al.*, 2002; Kelland, 2004). Their use as a predictive indicator of probable clinical activity has been validated for cytotoxicity. Efficacy of the drug in the human xenograft models can be measured in terms of tumor size or animal survival (Kerbel, 2003). Differences in tumor size between treated (T) and control (C) groups of aminals are most commonly expressed by the percent change in tumor size (T/C%) or percentage of growth inhibition (100% - T/C%).

Chapter 2

Materials and Methods

2.1 Phytochemicals

The pure compound 3α-hydroxylup-20(29)-ene-23,28-dioic acid (HLDA) and the ethyl acetate fraction were prepared from *Schefflera heptaphylla*. They were kindly provided by Dr. Yaolan Li (Jinan University, Guangzhou, P. R. China). The detailed extraction protocols were described by Li (Li, 2005). Betulinic acid (both were purchased from Sigma, St. Louis, MO) were used in this study as a comparison of the antiproliferative activities of HLDA and the ethyl acetate fraction. The chemical structures of HLDA and betulinic acid were shown in Fig. 1.1.

2.2 Chemicals, Cell Lines and Culture Conditions

All the chemicals and culture media were purchased from Sigma, unless otherwise stated. Six cell lines of different histotypes were used in this study. They included A375, human melanoma cells; HEp2, human laryngeal carcinoma cells; HepG2, human hepatocellular carcinoma cells; MCF-7, human breast carcinoma cells; PC-3, human prostate carcinoma cells and Vero, monkey normal kidney cells. All the cell lines were obtained from American Type Culture Collections (ATCC), Rockville, MD. A375,

HEp2 and Vero cells were maintained in Dulbecco's modified Eagle's medium; HepG2 and MCF-7 cells were maintained in RPMI 1640 medium, and PC-3 was maintained in F12K medium. All media were supplemented with 0.2% sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (all from Gibco-BRL, Gaithersburg, MD). All cell lines were kept under a fully humidified atmosphere at 37 ℃ in 5% CO_2. The cells were passaged two to three times a week in 25 cm^2 tissue culture flasks (Sarstedt, Nümbrecht, Germany). Upon passages, the cells were detached from the flasks by treatment with 1 ml of Trypsin-Versene solution (containing 0.25% trypsin and 0.02% EDTA) for 1 min, and the reaction was then stopped by the corresponding medium. The cells were washed once by phosphate-buffered saline (PBS), and spinned at 514 × *g* for 5 min (Beckman, Allegra™ 6R centrifuge). The cell pellet was resuspended in the medium, and the number of viable cells was then counted under a hemacytometer after staining with 0.4% trypan blue solution. Cells were generally seeded at a density of 2×10^5 cells/ml for each new passage.

2.3 Determination of *in vitro* antiproliferative effects of HLDA and the ethyl acetate fraction from *S. heptaphylla* on human cancer cells

2.3.1 MTT assay

Effects of HLDA and the ethyl acetate fraction on the proliferation and viability of A375, HEp2, HepG2, MCF-7 and PC-3 cancer cells and normal kidney Vero cells were determined by MTT assay.

Stock sample solutions were prepared by dissolving HLDA and the ethyl acetate fraction in dimethyl sulfoxide (DMSO). The final test concentrations of the sample were prepared by diluting the stock solution with the corresponding culture medium before each experiment. The final concentration of DMSO did not exceed 0.5% in every experiment. To determine the antiproliferative activity and the general cytotoxic effects of HLDA and the ethyl acetate fraction, the cells were seeded in 96-well flat-bottomed microtiter plates (IWAKI, Chiba, Japan) at a density of 2.5×10^3 viable cells/well/100 µl. After 24 h, the confluent cell cultures were incubated with 0, 12.5, 25, 50 and 100 µg/ml of HLDA and 0, 12.5, 25, 50, 100 and 200 µg/ml of ethyl acetate fraction for 72 h. After treatment, the cells were incubated with 10 µl of freshly prepared MTT solution (5 mg/ml in PBS) at 37 ℃ for 4 h. The precipitated formazan was solubilized with 150 µl of 0.04 N HCl-isopropanol, and the optical densities at 570 nm were determined by microplate reader. All test concentrations were done in

triplicate wells. The percentage inhibition of cell growth was calculated by the following equation:

$$\left(1 - \frac{\text{Average absorbance of treatment group}}{\text{Average absorbance of control group}}\right) \times 100\%$$

2.4 Determination of the *in vitro* antiproliferative mechanisms of HLDA and the ethyl acetate fraction from *S. heptaphylla* in human melanoma A375 cells

2.4.1 Flow cytometric analysis

Effects of HLDA and the ethyl acetate fraction on cell cycle distribution of A375 cells were analyzed by flow cytometer. Human melanoma A375 cells were seeded in 25 cm^2 sterile polystyrene tissue culture flasks at 2.5 × 10^4 cells/ml. The cells were either treated with the ethyl acetate fraction or HLDA. After incubation in the absence or presence of the IC_{50} of the ethyl acetate fraction or HLDA for 72 h, 2 × 10^6 A375 cells were harvested, and washed twice with PBS. The cells were then fixed overnight at -20 ℃ in 70% ethanol. The fixed cells were washed with PBS twice, and were then incubated in dark for 30 min in 1 ml PBS solution, containing 1 mg/ml ribonuclease and 10 μg/ml propidium iodide (PI) (Sigma). The red fluorescence of DNA-bound PI in individual cells was measured at >625 nm, anaylsed with Beckman Coulter Epics XL-MCL flow cytometer (Miami, FL). Cell cycle distribution was analyzed using

MultiCycle software (Phoenix Flow Systems, San Diego, CA)

To study the roles of caspases in HLDA and the ethyl acetate fraction-induced apoptosis, A375 cells were preincubated with or without 20 μM of the caspase inhibitor for 30 min prior to treatment with the IC_{50} of HLDA (i.e. 23 μg/ml) or ethyl acetate fraction (i.e. 46 μg/ml) for 72 h. The caspase inhibitors used were general caspase inhibitor (Z-VAD-FMK), caspase-8 inhibitor (Ac-IETD-CHO), and caspase-9 inhibitor (Z-LEHD-FMK) (all of them were purchased from PharMingen, San Diego, CA). The cells were then subjected to DNA content analysis by flow cytometry.

2.4.2 Western blot analysis

Effects of HLDA and the ethyl acetate fraction on expression of apoptosis-regulatory proteins were measured semi-quantitatively by SDS-PAGE and Western blot analysis. A375 cells were seeded in 25 cm^2 sterile polystyrene tissue culture flasks at 2.5×10^4 cells/ml. After 24 h of acclimatization, the cells were treated in the absence or presence of the IC_{50} of HLDA or the ethyl acetate fraction for 72 h.

After treatment, 2×10^6 cells were harvested for protein extraction. The cells were washed twice with ice-cold PBS with centrifugation at 4 ℃. The cell pellet was then resuspended and homogenized in 49 μl ice-cold lysis buffer with 1 μl protease inhibitor (Calbiochem, San Diego, CA). The cell lysate was then transferred to a microcentrifuge

tube and incubated on ice for 45 min. The cell lysate was then centrifuged for 15 min at 4 ℃ after the incubation. Finally, supernatant containing the cellular proteins was collected and frozen at -20 ℃.

The protein content was measured by bicinchoninic acid (BCA) assay. One microliter of the cell lysate was diluted with 49 μl ultrapure water in a microcentrifuge tube. A standard curve was prepared using 50 μl of 0, 25, 50, 100, 200 and 400 μg/ml bovine serum albumin (BSA) solution. One milliliter of BCA assay working reagent, containing 1 ml Reagent A with 20 μl Reagent B (Pierce, Rockford, IL), was then added to the microcentrifuge tube and incubated for 30 min at 37 ℃. After the incubation, all the tubes were cooled to room temperature. Absorbance of each tube was then measured at 562 nm using Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany), and protein content of the cell lysate was determined from the standard curve.

Ten to twenty microliters of cellular proteins (30-80 μg) were then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The proteins were mixed with equal volume of 2× loading buffer, containing Tris-HCL, DTT, SDS, bromophenol blue and glycerol, in a microcentrifuge tube. The proteins were then denatured by incubation for 5 min at 95 ℃. The proteins were then cooled so that 30 to 80 μg were resolved by 13% SDS-PAGE. The resolved protiens were then

transferred to a Hybond™-C nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK). The membrane was then blocked with 0.2% Aurora® blocking reagent (ICN Biomedicals, Ohio, USA) or 0.5% non-fat dry milk, with 0.1% Tween-20 solution. The blocked membrane was the incubated overnight with the primary antibody, including mouse monoclonal antibodies to Bcl-2 and Bax (BD Biosciecnces PharMingen), mouse monoclonal antibodies to caspase-8 and β-actin (Cell Signaling Technology, Beverly, MA), and rabbit polyclonal antibodies to PARP, Bcl-XL, p53, Bid, Bak and caspase-9 (Cell Signaling Technology).

After the incubation, the membrane was washed, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG polyclonal antibodies (Cell Signaling Technology) for 1 h, with shaking at room temperature. Finally, the membrane was washed, incubated with 10 ml LumiGLO Substrate (Cell Signaling Technology), and then exposed to X-ray film.

2.5 Determination of the *in vivo* anticancer effects of the ethyl acetate fraction from *S. heptaphylla*

2.5.1 Determination of cancer chemopreventive effect of the ethyl acetate fraction with DMBA/TPA-induced skin carcinogenesis model

The cancer preventive effect of the ethyl acetate fraction was examined using a standard initiation-promotion protocol with 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as previously reported (Ding *et al.*, 2004) with some modifications.

Seven to eight-week-old female ICR mice were obtained from the Laboratory Animal Service Centre (LASEC) of the Chinese University of Hong Kong. All experiments were performed according to the guidelines approved by the Chinese University of Hong Kong Animal Experimentation Ethics Committee (AEEC). The mice were housed in the animal house of the Department of Biology, CUHK, under controlled laboratory conditions in rodent microisolator cages at 25 ± 2 ℃ with a 12 h light/dark cycle and were fed with a standard diet and tap water *ad libitum*. Mice were allowed to acclimatize in the laboratory for one week before experiments.

The mice were then randomly divided into six groups of 7 or 8. Dorsal skin of the mice was shaved with a surgical clipper one day before initiation. The mice were then exposed to a single dose of 300 nmol of DMBA in 200 μl of acetone. Seven days

following initiation, the mice were topically treated with 0, 0.4, 2, 4, 8 and 40 mg/ml of ethyl acetate fraction (in acetone) 30 min before promotion by exposure to 7.5 nmol of TPA in 200 μl of acetone. The ethyl acetate fraction and TPA were administered twice a week for a total 20 weeks. Incidence of papilloma-bearing mice and number of papillomas (>1 mm in diameter) were recorded once every 2 weeks. The cancer preventive effect of the ethyl acetate fraction was determined by comparing the fraction-treated groups with the untreated control group.

2.5.2 Determination of cancer therapeutic effect of the ethyl acetate fraction with athymic BALB/c nude mice model

Human melanoma A375 cells were implanted subcutaneously (s.c.) into athymic nude mice to study the cancer therapeutic effect of the ethyl acetate fraction.

Male athymic mice (BALB/c, nu/nu, 7 to 8 weeks old) were obtained from the LASEC, CUHK. The animals were housed in Room 403 of LASEC under clean and controlled room conditions in sterile rodent microisolator cages at 25 ± 2 ℃ with a 12 h light/dark cycle. Animals were fed with sterile rodent chow and water *ad libitum* and were acclimated in the laboratory for one week before experiment. All experiments were performed according to guidelines approved by the AEEC of CUHK.

A375 cells were kept in 175 cm^2 sterile tissue culture flasks and were maintained

under conditions as described in Section 2.2. Cells were detached from the flasks by treatment with 3 ml of Trypsin-Versene solution for 1 minute and the reaction was then stopped by DMEM medium. The cells were washed once by PBS and centrifugation at 514 × *g* for 5 min. The cell pellet was re-suspended in DMEM medium, and the number of viable cells was then counted under a hemocytometer after staining with 0.4% trypan blue solution. Cell suspension with a density of 2.5×10^7 cells/ml was prepared for inoculation into the nude mice.

Five million A375 cells were inoculated subcutaneously at the back of each athymic nude mice. The A375 solid tumors were allowed to grow for 10 days before the mice were separated randomly into five groups of six. The mice were then treated topically with 0, 2, 4, 8 and 40 mg/ml of ethyl acetate fraction in acetone once a day for consecutive 10 days. After treatment, the mice were sacrificed by cervical dislocation.

Tumors were then excised and measured in term of weight by an electronic balance. The inhibitory effect of the ethyl acetate fraction on the growth of A375 tumor was expressed as reduction of tumor weight in the fraction-treated groups when compared to the untreated control group.

2.6 Statistical Analysis

Except the data from animal studies, all the other data are presented as mean ± standard deviation (S.D.). Animal data are shown as mean ± standard error of measurement (S.E.M.). Difference in means between the control and experimental groups was compared by Student's *t*-test; $*p<0.05$, $**p<0.01$, and *** $p<0.001$. For caspase inhibitors analysis, variance between multiple groups was analysed by ANOVA, followed by Tukey's test, where $p < 0.05$ indicates statistically significant difference.

Chapter 3

Results

3.1 Effects of HLDA and the ethyl acetate fraction on viability and proliferation of different cancer cell lines by MTT assay

Effects of HLDA and the ethyl acetate fraction on cell viability and proliferation were studied in five human cell lines originated from different tissues by MTT assay. They included A375, melanoma cells; HEp2, laryngeal carcinoma cells; HepG2, hepatocellular carcinoma cells; MCF-7, breast carcinoma cells; and PC-3, prostate carcinoma cells.

After 72 h incubation with HLDA or the ethyl acetate fraction, viability and proliferation of all the tested cancer cell lines were inhibited dose dependently (Fig. 3.1 and Fig. 3.2). Treatment with 100 μg/ml of HLDA suppressed the viability and proliferation of A375, HEp2, HepG2, MCF-7 and PC-3 cells by 78.3%, 59.5%, 84.3%, 76.7% and 60.4% respectively, comparing to the control cells. Whereas, treatment with 200 μg/ml of ethyl acetate fraction suppressed the viability and proliferation of A375, HEp2, HepG2, MCF-7 and PC-3 cells by 94.0%, 77.9%, 79.6%, 88.9% and 76.1%, respectively.

The values of 50% inhibitory concentration on the viability and proliferation of

the five cancer cell lines (IC_{50}) were subsequently estimated from the dose-activity plots. The results were summarized in Table 3.1. The values of IC_{50} for A375, HEp2, HepG2, MCF-7 and PC-3 cells were 23, 90, 33, 40 and 86 μg/ml, respectively. On the other hand, the IC_{50} values for the ethyl acetate fraction on A375, HEp2, HepG2, MCF-7 and PC-3 cells were 46, 110, 52, 76 and 100 μg/ml, respectively. By comparing the IC_{50} values, A375 melanoma was apparently the most responsive cell line to the HLDA and ethyl acetate fraction treatments.

3.2 Effects of HLDA and the ethyl acetate fraction on cell cycle and apoptosis in A375 cells determined by DNA flow cytometry

Since the MTT studies showed that A375 melanoma was the most responsive cancer cell line to the reduction of viability and proliferation induced by HLDA or the ethyl acetate fraction, the associated mechanisms were further investigated in the cancer cell line.

The melanoma cells were incubated in the absence or presence of 23 μg/ml of HLDA or 46 μg/ml of the ethyl acetate fraction for 72 h. Sub-G_1 peak, representing the apoptotic cells elevated prominently in the treated cells, in comparison of the control cells (Fig. 3.3). The apoptotic cells were then measured, and were expressed as proportion of the total analyzed cells. Both HLDA and the ethyl acetate fraction,

induced apoptosis in the cancer cells, so that the apoptotic cells with fragmented DNA elevated by 24-fold and 12-fold, respectively, of the control level (Fig. 3.4).

Besides apoptosis induction, results from the DNA-flow cytometric study also showed that the ethyl acetate fraction also altered cell-cycle progression of the melanoma cells. The extract seemed to arrest S-phase cells so that they increased from 17.0% of the cells in cell cycle to 36.8%; G_2/M cells also elevated from the control 9.7% to 17.9%. G_0/G_1 cells, on the other hand, decreased from 73.8% to 42.2%. However, HLDA did not modulate the cell-cycle progression of the melanoma cells significantly after 72 h of incubation. All these findings suggested that the fraction-induced growth inhibition on the melanoma cells seemed to be mediated by both apoptosis induction and cell-cycle arrestment, while the HLDA-induced growth inhibition was mediated by apoptosis only.

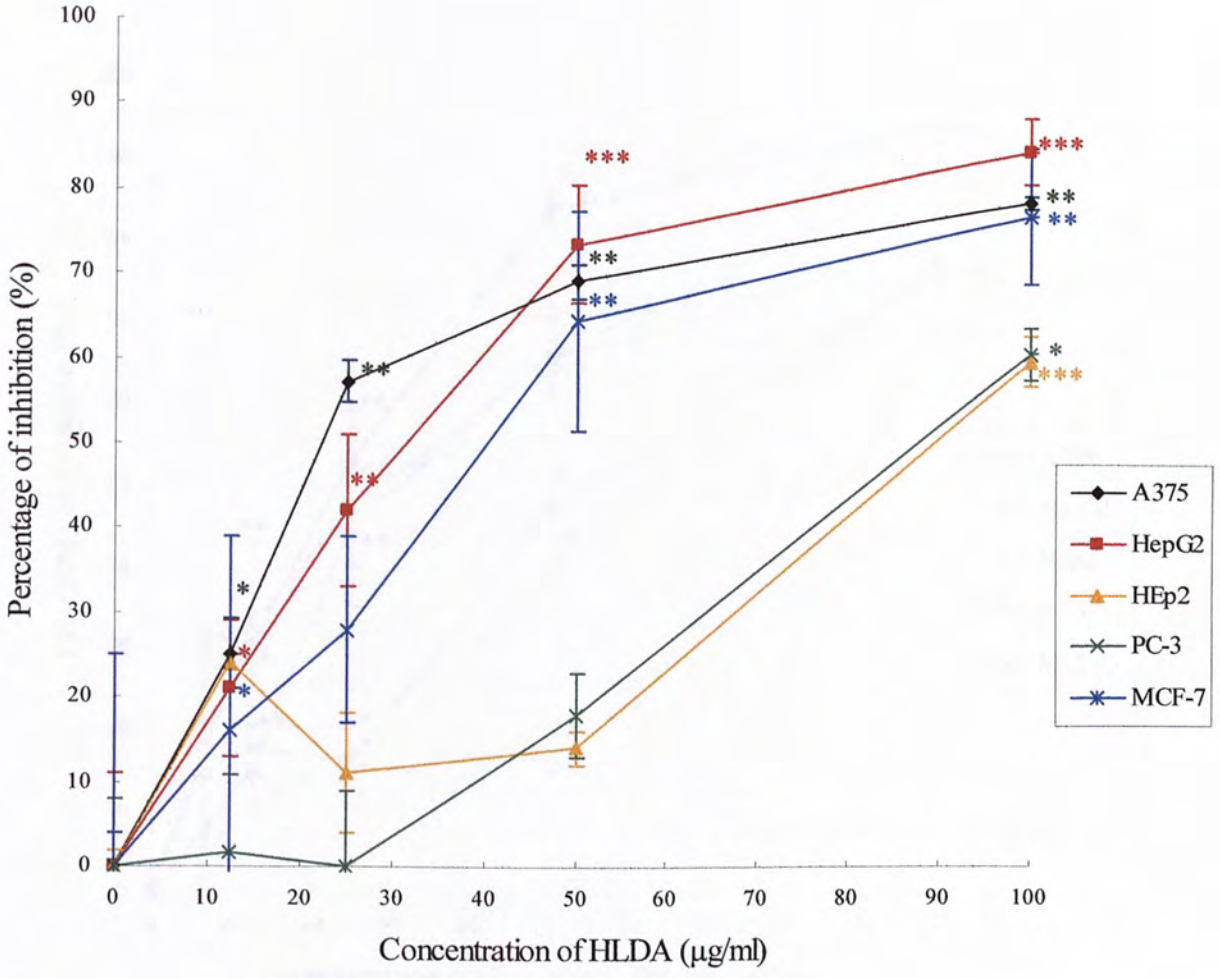


Fig. 3.1 Effects of HLDA on the proliferation and viability of A375, HepG2, HEp2, PC-3 and MCF-7 cells determined by MTT assay. Results are expressed as mean ± S.D. of three independent experiments. *, ** and *** represent statistical significance at $p<0.05$, $p<0.01$ and $p<0.001$ respectively, determined by Student's *t*-test.

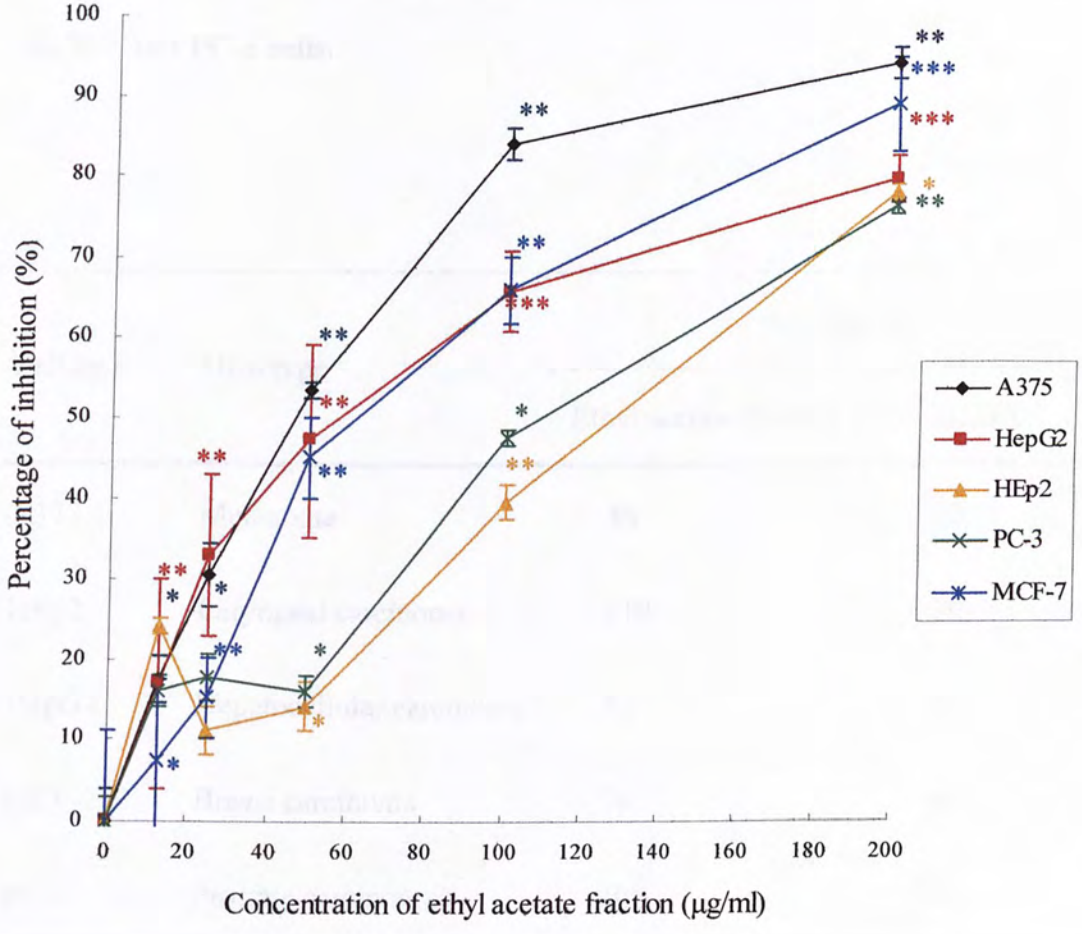


Fig. 3.2 Effects of the ethyl acetate fraction on the proliferation and viability of A375, HepG2, HEp2, PC-3 and MCF-7 cells determined by MTT assay. Results are expressed as mean ± S.D. of three independent experiments. *, ** and *** represent statistical significance at $p<0.05$, $p<0.01$ and $p<0.001$ respectively, determined by Student's *t*-test.

Table 3.1 The values of 50% inhibitory concentration (IC_{50}) of the ethyl acetate fraction and HLDA on the viability and proliferation of A375, HEp2, HepG2, MCF-7 and PC-3 cells.

Cell line	Histotype	IC_{50} (μg/ml)	
		Ethyl acetate fraction	HLDA
A375	Melanoma	46	23
HEp2	Laryngeal carcinoma	110	90
HepG2	Hepatocellular carcinoma	52	33
MCF-7	Breast carcinoma	76	40
PC-3	Prostate carcinoma	100	86

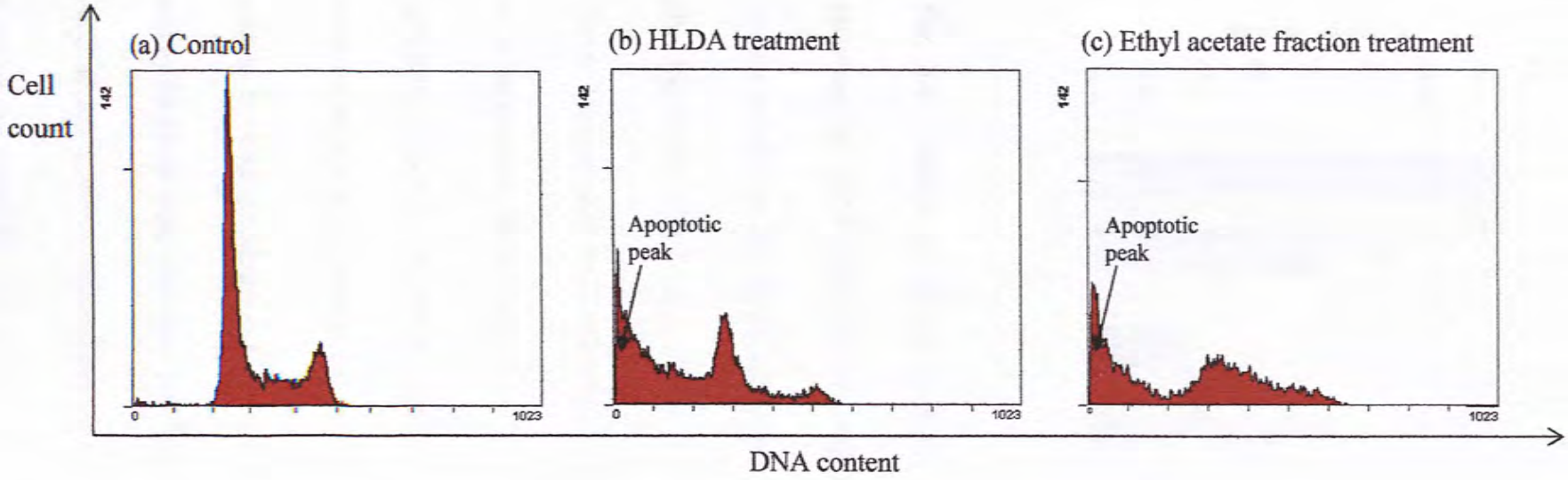


Fig. 3.3 Representative DNA histograms showing the effects of HLDA and the ethyl acetate fraction on cell cycle and apoptosis in A375 cells. The melanoma cells were treated for 72 h, (a) in the absence, i.e. control, or in the presence of (b) 23 μg/ml of HLDA, or (c) 46 μg/ml of the ethyl acetate fraction. Both HLDA and the ethyl acetate fraction elevated sub-G_1 or apoptotic cells prominently. The DNA histograms were obtained by flow cytometric analysis of the propidium iodide-stained cells at 72 h of treatment. The histograms shown above are representatives of three independent experiments. Numeric data are shown in Fig. 3.2.

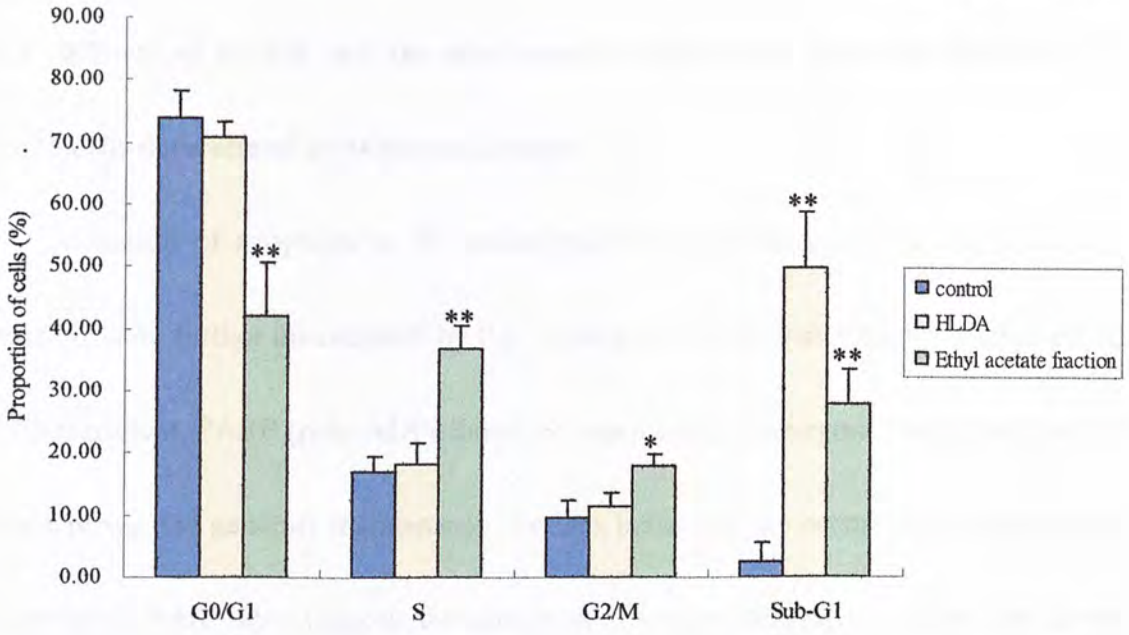


Fig. 3.4 Effects of HLDA and the ethyl acetate fraction on cell cycle and apoptosis in A375 cells. Numeric data showing the effects of HLDA and the ethyl acetate fraction on cell cycle and apoptosis in A375 cells measured by flow cytometer after incubation with the IC_{50} of HLDA or the IC_{50} of the ethyl acetate fraction for 72 h. The melanoma cells were treated for 72 h, either in the absence, i.e. control (blue bars) or in the presence of HLDA (yellow bars) or the ethyl acetate fraction (green bars). The apoptotic cells were measured using the control software of the flow cytometer, and were expressed as proportion of the total analyzed cells. Both HLDA and the ethyl acetate fraction elevated sub-G_1or apoptotic cells prominently. The cells in different cell cycle phase were expressed as proportion of the cells in cell cycle. In addition, the ethyl acetate fraction elevated the cells in S and G_2/M phases whereas HLDA did not. *p<0.05 and **p<0.01, compared to the control level by Student's t-test.

3.3 Effects of HLDA and the ethyl acetate fraction on apoptosis induction in A375 cells determined by Western blotting

Induction of apoptosis in the melanoma cells by HLDA and the ethyl acetate fraction were further investigated by the western-blot study that PARP was cleaved at 72 h treatment. PARP [poly(ADP-ribose) polymerase] is an enzyme that is involved in DNA repair and genomic maintenance. Besides indicating the occurrence of apoptosis, cleavage of PARP also suggests the activation of proteolytic caspases in the cell death process. During the execution phase of apoptotic cell death, proteolysis of the intact form of PARP (cleaved from 116 kDa into 89 kDa) results in the loss of normal PARP function, which facilitates cellular disassembly. As shown in Fig. 3.5, after 72 h incubation with HLDA or the ethyl acetate fraction, PARP cleavage was observed in the treated cells, in comparison to the intact PARP in the control cells. Results from the immunoblot suggest that either HLDA or the ethyl acetate fraction induces apoptosis in the melanoma cells possibly via activation of caspases.

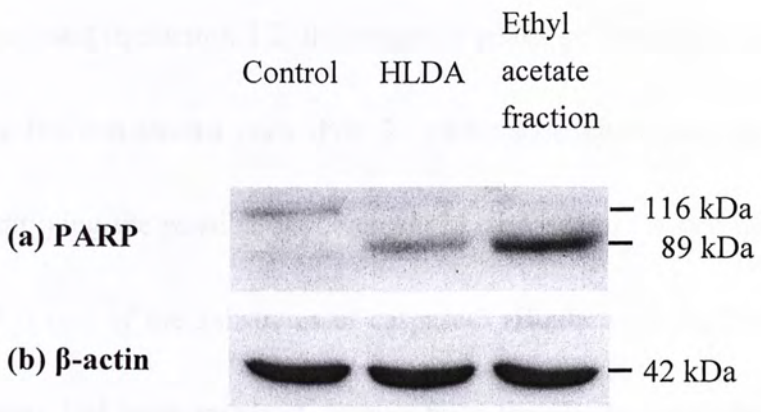


Fig. 3.5 Representative immunoblot showing the effects of HLDA and ethyl acetate fraction on PARP protein. PARP cleavage from 116 kDa to 89 kDa was observed at 72 h treatment with HLDA or the ethyl acetate fraction, suggesting that apoptosis was induced in the melanoma possibly via activation of caspases. β-actin was included as loading control to normalize expressions of the PARP protein.

3.4 Effects of HLDA and ethyl acetate fraction on caspases in A375 cells

As mentioned in Section 3.2, the apoptotic peaks generated in the HLDA and the ethyl acetate fraction-treated cells (Fig. 3.3) were associated with cleavage of PARP (Fig. 3.5), implying the possible involvement of caspases in the cell death mechanisms since PARP is one of the substrates of caspase-3 (Boulares *et al.*, 2002). To find out which caspases had been involved, lysates from the HLDA- and the fraction-treated A375 cells were immunoblotted with specific antibodies against caspase-8 and caspase-9, which are the initiator caspases of the extrinsic and the intrinsic apoptotic pathways, respectively. As shown in Fig. 3.6*a*, processing of procaspase-8 into the active p43 and p41 subunits was more prominent in HLDA- and the fraction-treated A375 cells than in the control cells. Similarly, cleavage of procaspase-9 into active p37 and p35 fragments was evident upon treatment with either HLDA or the fraction (Fig. 3.6*b*). These data suggested that both the extrinsic and the intrinsic apoptotic pathways had been elicited by HLDA and the ethyl acetate fraction in the A375 cells.

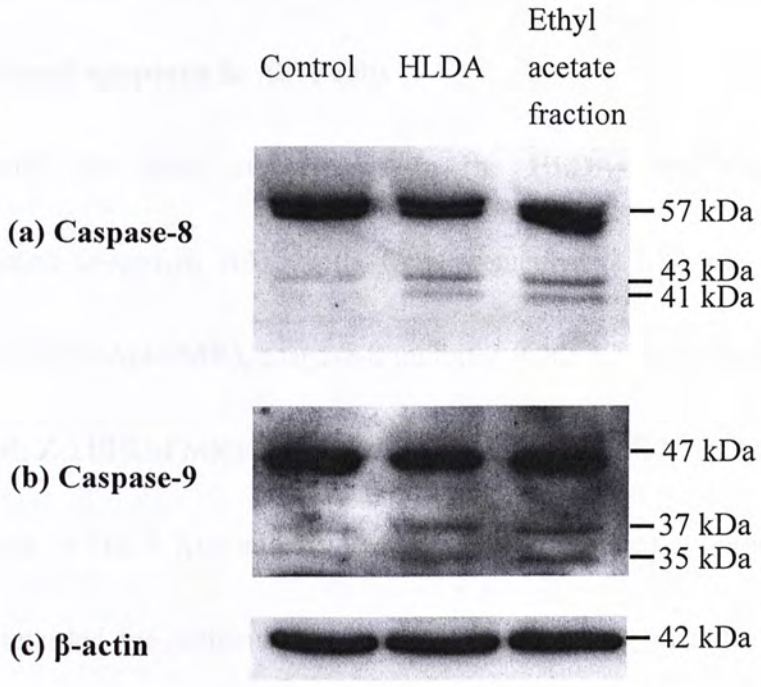


Fig. 3.6 Representative immunoblots showing the effects of HLDA and the ethyl acetate fraction on caspase-8 and caspase-9. Lysates from the HLDA- and the fraction-treated A375 cells were analysed for caspases by Western blotting. Cleavage of caspase-8 and caspase-9 into their active subunits was observed in both the HLDA- and the fraction-treated cells. β-actin was included as loading control.

3.5 Effects of caspase inhibitors on the HLDA- and the ethyl acetate fraction-induced apoptosis in A375 cells

To verify the roles of caspases in the HLDA- and the ethyl acetate fraction-induced apoptosis, A375 cells were preincubated with the general caspase inhibitor (GCI; Z-VAD-FMK), caspase-8 inhibitor (C8I; Ac-IETD-CHO) or caspase-9 inhibitor (C9I; Z-LEHD-FMK) prior to the treatment with HLDA or the fraction.

As shown in Fig. 3.7*a-c* and Fig. 3.8, the HLDA-induced apoptosis in A375 was completely retarded by preincubation with GCI, suggesting that the HLDA-induced apoptosis was executed solely by the caspase-dependent events. In contrast, preincubation with GCI could only partially inhibit the ethyl acetate fraction-induced apoptosis (Fig. 3.9*a-c* and Fig. 3.12), suggesting some of the apoptotic cells were resulted from caspase-independent events.

As shown in Fig. 3.7*d&e* and Fig. 3.8, the HLDA-induced apoptosis in A375 cells was partially inhibited after preincubation with C8I or C9I, indicating that caspase-8 and -9 had been playing important roles in the HLDA-induced apoptosis. On the contrary, the apoptosis induced by the ethyl acetate fraction could only be retarded by caspase-8 or caspase-9 inhibitor to a lesser extent, suggesting that the initiator caspases had played lesser significant functions in the cell death process (Fig. 3.9*d&e* and Fig. 3.10).

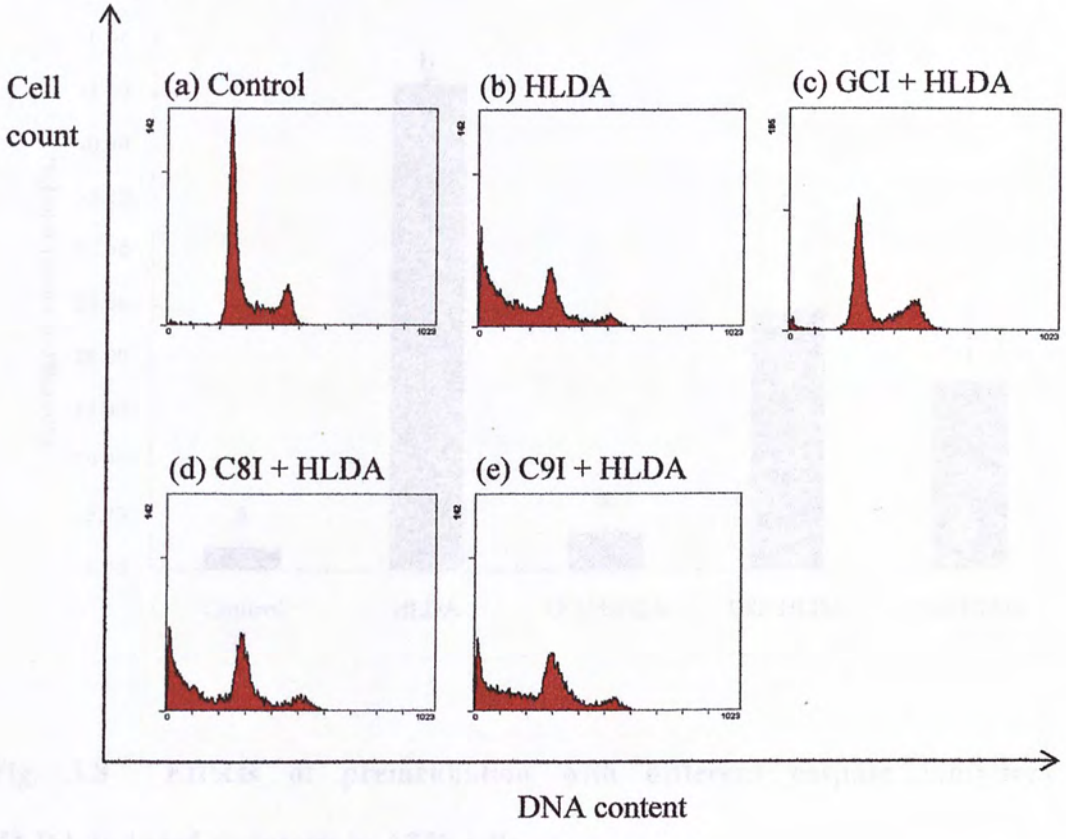


Fig. 3.7 Representative DNA histograms showing the effects of different caspase inhibitors on HLDA-induced apoptosis.

Human A375 cells were preincubated with or without different caspase inhibitors for 30 min prior to treatment with HLDA for 72 h. The cells were then subjected to DNA-flow cytometry analysis.

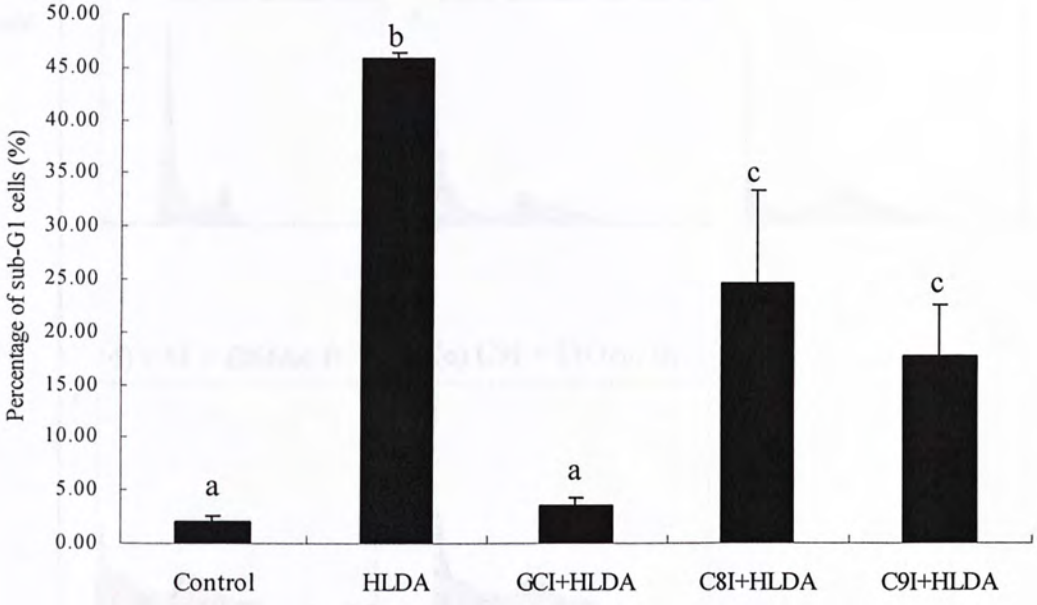


Fig. 3.8 Effects of preincubation with different caspase inhibitors on HLDA-induced apoptosis in A375 cells

A375 cells were preincubated with or without different caspase inhibitors prior to the addition of HLDA. The cells were then subjected to DNA-flow cytometry analysis. The percentage of sub-G_1 cells (i.e. apoptotic cells) are presented numerically and are expressed as mean ± S.D. Data were analysed by ANOVA, followed by Tukey's test. Bars with the same letter indicate no significant differences (p>0.05).

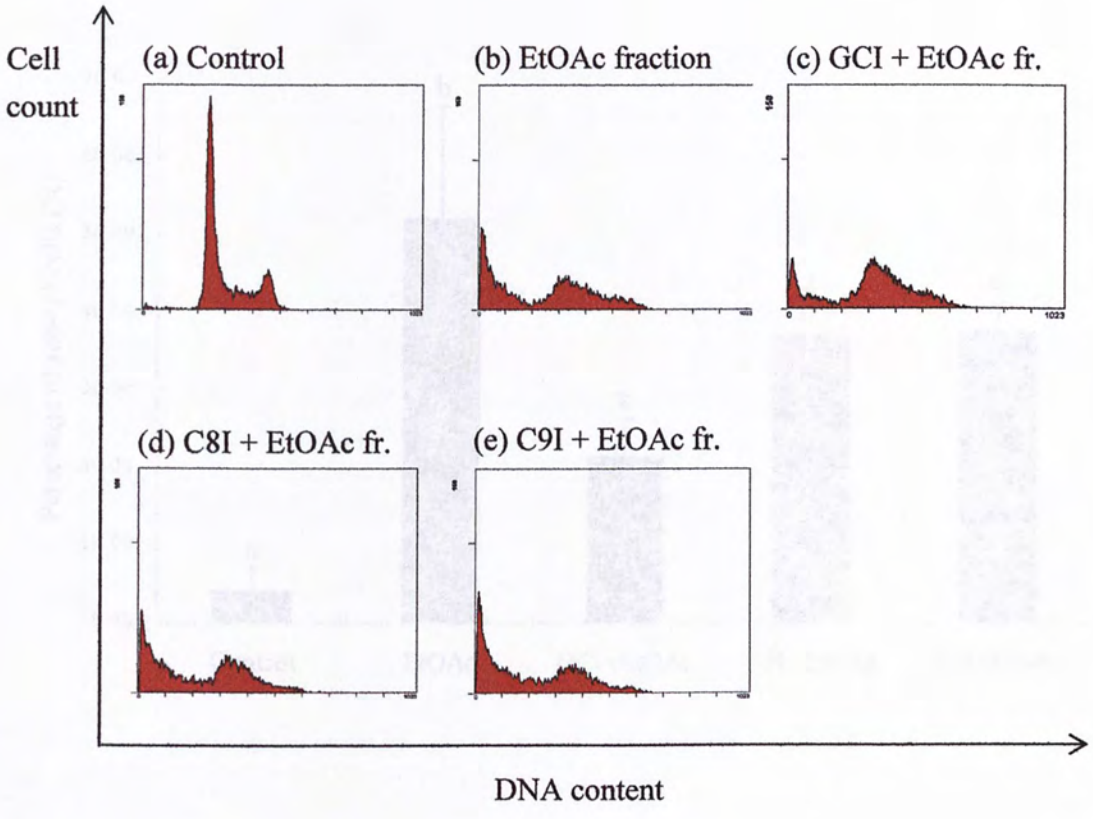


Fig. 3.9 Representative DNA histograms showing the effects of preincubation different caspase inhibitors on the ethyl acetate fraction-induced apoptosis.

Human A375 cells were preincubated with or without different caspase inhibitors for 30 min prior to treatment with the ethyl acetate fraction for 72 h. Cells were then subjected to DNA-flow cytometry analysis.

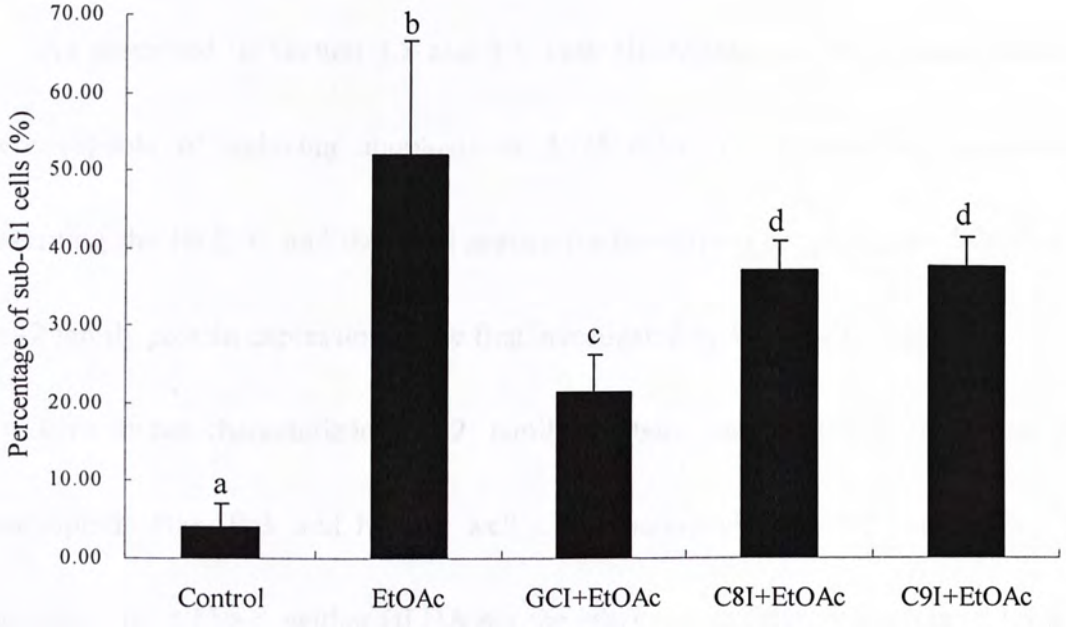


Fig. 3.10 Effects of preincubation with different caspase inhibitors on ethyl acetate fraction-induced apoptosis in A375 cells

A375 cells were preincubated with or without different caspase inhibitors prior to the addition of the ethyl acetate fraction. Cells were then subjected to DNA-flow cytometry analysis. The percentage of sub-G_1 cells (i.e. apoptotic cells) are presented numerically and are expressed as mean ± S.D. Data were analysed by ANOVA, followed by Tukey's test. Bars with the same letter indicate no significant differences (p>0.05).

3.6 Effects of HLDA and the ethyl acetate fraction on the expression of Bcl-2 family proteins in A375 cells

As described in Section 3.2 and 3.3, both HLDA and the ethyl acetate fraction were capable of inducing apoptosis in A375 cells. To explore the mechanisms governing the HLDA- and the ethyl acetate fraction-driven apoptosis, modulations of Bcl-2 family protein expressions were first investigated by Western blot analysis.

Five better-characterized Bcl-2 family proteins were studied, including the proapoptotic Bax, Bak and Bid, as well as the antiapoptotic Bcl-2 and Bcl-X_L. As shown in Fig. 3.11*a-c*, neither HLDA nor the ethyl acetate fraction modulated level of the proapoptotic Bcl-2 family proteins tested in this study. On the other hand, both HLDA and ethyl acetate fraction depleted level of the antiapoptotic Bcl-X_L (Fig. 3.11*e*). Interestingly, another antiapoptotic member, Bcl-2, was only depleted by the ethyl acetate fraction, but not by HLDA (Fig. 3.11*d*), suggesting that the ethyl acetate fraction may contain molecules other than HLDA that have additional modulatory effect.

Taken together, these results indicate that HLDA and the ethyl acetate fraction act at least partially by decreasing the ratio of antiapoptotic to proapoptotic protein expression in A375 cells, through depletion of Bcl-2 and Bcl-X_L. This may be one of the possible mechanisms to address the capability of HLDA and the ethyl acetate

fraction from *S. heptaphylla* to induce apoptosis in A375 cells.

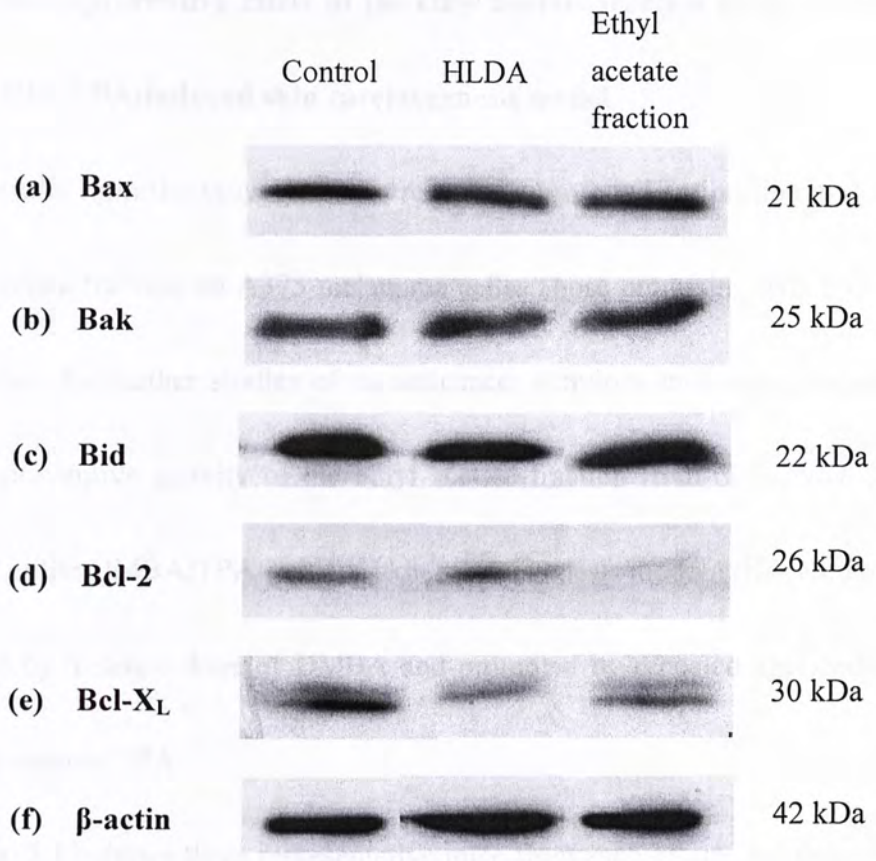


Fig. 3.11 Representative immunoblots showing the effects of HLDA and the ethyl acetate fraction on expression of Bcl-2 family proteins. Human melanoma A375 cells treated with or without HLDA or the fraction were analysed for Bcl-2 family protein expression by Western blotting. β-actin was included as loading control. HLDA did not modulate protein level of Bax, Bak, Bid and Bcl-2, while Bcl-X_L protein was significantly depleted. On the other hand, levels of Bcl-X_L and Bcl-2 were depleted after treatment with the ethyl acetate fraction.

3.7 Chemopreventive effect of the ethyl acetate fraction from *S. heptaphylla* on the DMBA/TPA-induced skin carcinogenesis model

Results from the *in vitro* studies revealed a profound antiproliferative effect of the ethyl acetate fraction on A375 melanoma cells. Those promising data provided a solid foundation for further studies of its anticancer activities in animal models. Thus, the cancer preventive activity of the ethyl acetate fraction from *S. heptaphylla* was first studied in the DMBA/TPA-induced skin carcinogenesis model. The carcinogenesis was initiated by a single dose of DMBA and promoted by repeated applications with the tumor promoter TPA.

Fig. 3.12 shows three representative mice from each group. As clearly depicted in the photographs, papillomas on the mice of the control group grew tremendously and were well vascularized, whereas those on the mice of the fraction-treated groups were growth-arrested and desiccated, or even not found at all.

The mortality rate and the papilloma incidence of mice in the control and treatment groups are summarized in Table 3.2. For the entire period of treatment, no mice were found dead, indicating that the ethyl acetate fraction is relatively safe when used topically. Treatments with low doses and high doses of the ethyl acetate fraction were led to dramatic decreases in both the number of papilloma-bearing mice and the number of papilloma on mice. Surprisingly, the ethyl acetate fraction was capable of

completely retarding papilloma development in the group that received the dose of 40 mg/ml. Moreover, the papilloma incidence-time plot showed that the ethyl acetate fraction retarded the papilloma growth dose-dependently (Fig. 3.13).

(a) Control group

(d) 4 mg/ml

(b) 0.4 mg/ml

(e) 8 mg/ml

(c) 2 mg/ml

(f) 40 mg/ml

Fig. 3.12 Photographs showing representative ICR mice treated with different dosages of the ethyl acetate fraction. In this DMBA/TPA-induced skin carcinogenesis model, skin papilloma development was initiated on the back of ICR mice by a single dose of DMBA. The promotion stage of the carcinogenesis was performed by repeated applications of TPA for 20 weeks. During the promotion period, the mice were topically treated with different concentrations of the ethyl acetate fraction 30 min after TPA application.

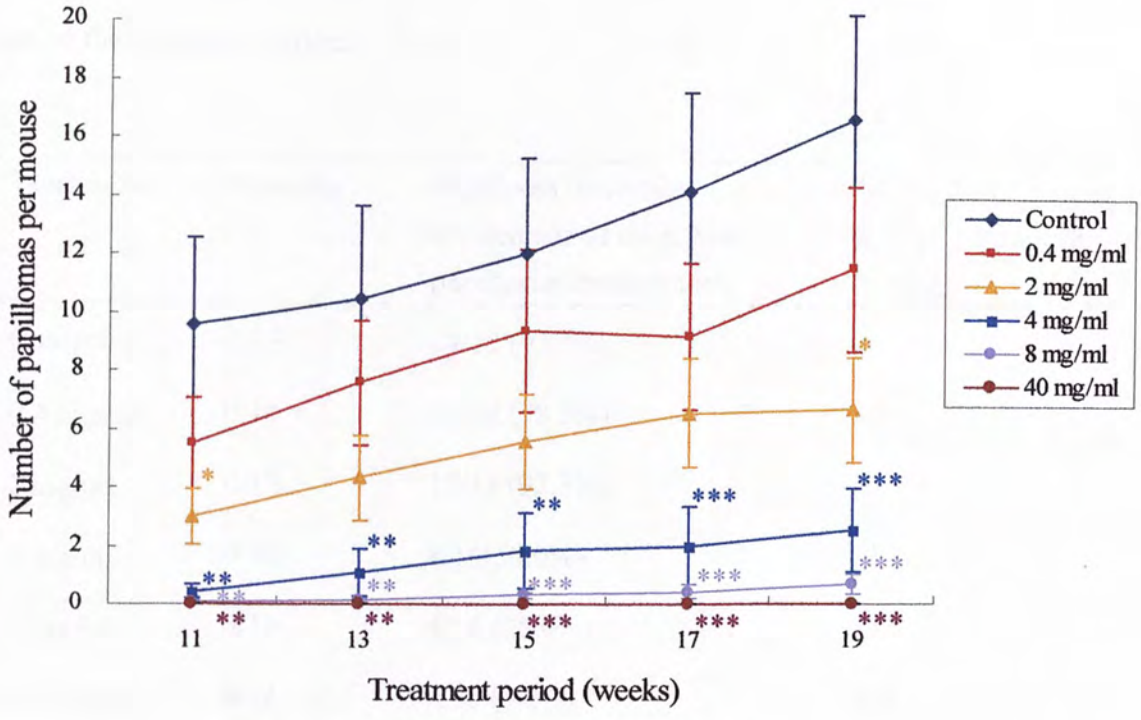


Fig. 3.13 Effect of the ethyl acetate fraction on papilloma development in the DMBA/TPA model. Number of papillomas (>1 mm) was recorded every 2 weeks starting from week 11. The results are presented as the mean number of papillomas per mouse ± S.E.M. *, **, and *** represent statistically significant difference from the control group at $p<0.05$, $p<0.01$ and $p<0.001$ respectively, determined by Student's *t*-test.

Table 3.2 Effects of different doses of the ethyl acetate fraction on DMBA/TPA induced papilloma development in ICR mice. The data showed were collected at the end of the treatment period.

Treatments	Mortality	Papilloma incidence (percentage of mice with papilloma development)	Actual dose applied per mouse (mg/kg)
Control	0/15	14/15 (93.3%)	0
0.4 mg/ml	0/14	11/14 (78.6%)	3.2
2 mg/ml	0/13	12/13 (92.3%)	16
4 mg/ml	0/16	8/16(50.0%)	32
8 mg/ml	0/16	4/16 (25.0%)	64
40 mg/ml	0/14	0/16 (0.0%)	320

3.8 Chemotherapeutic effect of the ethyl acetate fraction from *S. heptaphylla* on A375 xenograft in athymic nude mice

Since human xenograft model is one of the best models for predicting drug efficacy in clinical settings (Naoe *et al.*, 1998), antitumor activity of the ethyl acetate fraction against A375 xenograft in athymic nude mice was examined. The mice bearing A375 solid tumors were treated topically with different doses of the ethyl acetate fraction once daily for consecutive 10 days. At the end of treatment period, the tumors were excised and measured for their weight and volume. Three representative mice from each group are shown in Fig. 3.14. The ethyl acetate fraction treatment induced tumor regression significantly. The fraction induced regression in the weight (Fig. 3.15) of the xenograft. Even at dose as low as 2 mg/ml, the tumor weight and volume were reduced by >70% of the control level, indicating that the ethyl acetate fraction was extremely effective in controlling the growth of A375 xenograft *in vivo*. It is noteworthy that a mouse was found dead in the group that received the highest dosage of the ethyl acetate fraction (i.e. 40 mg/ml). However, a larger sample size is required to confirm whether that the death was attributed to the ethyl acetate fraction treatment or not.

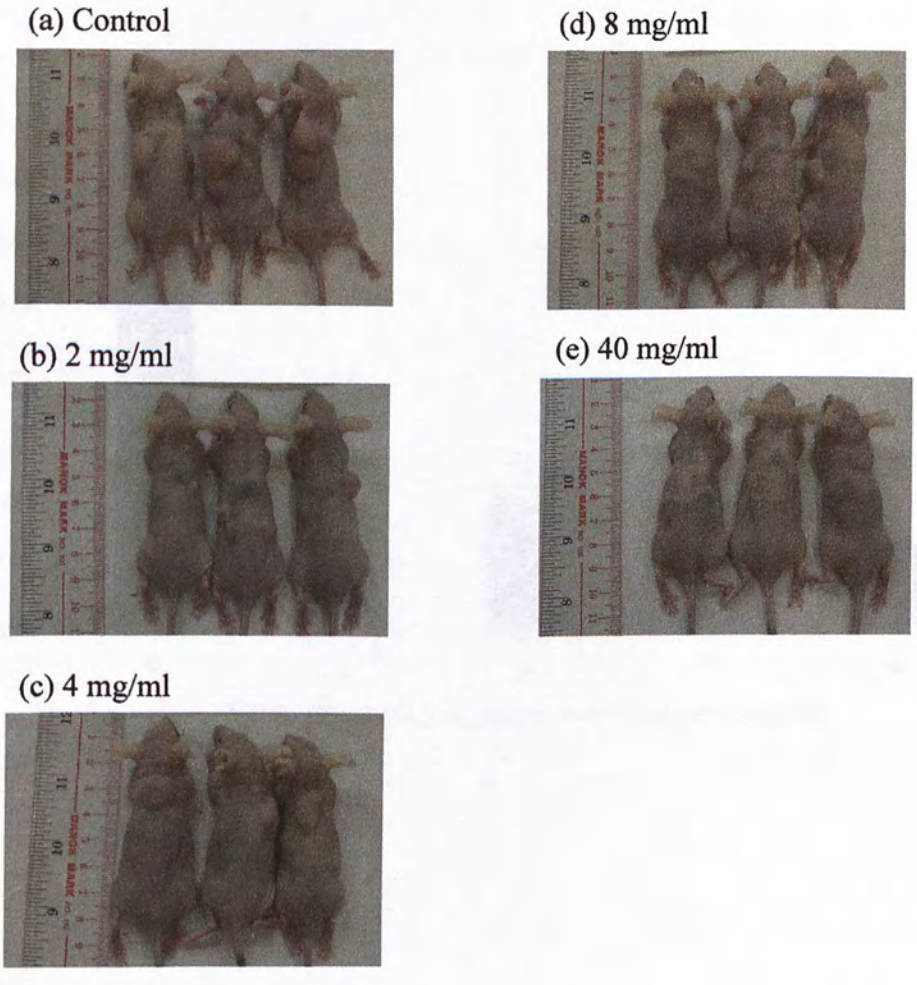


Fig. 3.14 Photographs showing representative athymic mice bearing A375 solid tumor treated with different dosages of the ethyl acetate fraction. Athymic mice were inoculated with human melanoma A375 cells subcutaneously. Ten days after inoculation, the mice were treated topically with the different dosages of the ethyl acetate fraction once daily for 10 consecutive days. The A375 solid tumors were allowed to grow for 10 more days before sacrificed.

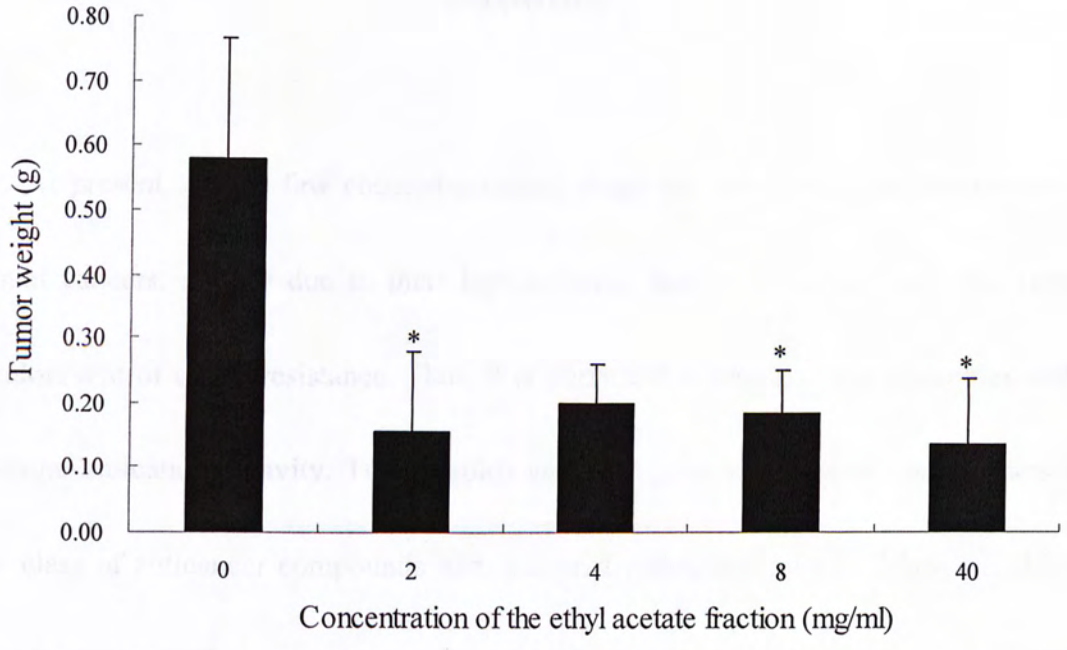


Fig. 3.15 Effect of the ethyl acetate fraction on the weight of A375 xenograft. The results are presented as the mean tumor weight ± S.E.M. * represents significant difference from the control group at $p<0.05$, determined by Student's *t*-test.

Chapter 4

Discussion

At present, only a few chemotherapeutic drugs are effective in the treatment of human cancers, mainly due to their high toxicity, lack of specificity and the rapid development of chemoresistance. Thus, it is important to identify new molecules with potential anticancer activity. Triterpenoids and their glycoside derivatives represent a new class of anticancer compounds with potential anticancer activity. Many of which have been reported as cancer-specific cytotoxic compounds (Pedersen *et al.*, 2002; Sarek *et al.*, 2003). In particular, betulinic acid, a pentacyclic triterpene, was discovered in 1995 as a selective inhibitor of human melanoma (Pisha *et al.*, 1995). This compound is currently under preclinical evaluation in the National Cancer Institute of the United States.

We have recently identified a pure compound, 3α-hydroxy-lup-20(29)-ene-23,28-dioic acid (HLDA), from *Schefflera heptaphylla* in an antiviral study (Li *et al.*, 2004). It was isolated for the first time from *Schefflera heptaphylla* by Adam *et al.* (1982), but its biological activity has never been documented. The chemical structure of HLDA is highly similar to that of betulinic acid, except the carboxyl group at carbon-28 of HLDA is replaced by a methyl group in the

latter (Fig. 1.1). This prompted us to investigate whether HLDA exhibits antiproliferative activity as well. In addition, since Kuo *et al.* (2002) reported strong anticancer activity of the ethyl acetate fraction from *S. taiwaniana*, the ethyl acetate fraction from *S. heptaphylla*, from which HLDA is derived, was also evaluated for its antiproliferative activity in this study.

By using the MTT assay, the inhibitory activities of HLDA and the ethyl acetate fraction on cell proliferation and viability were evaluated in five cancer cell lines of different histotypes. Growth of all the cancer cells was inhibited by HLDA and the ethyl acetate fraction dose-dependently. A375 melanoma was found to be the most sensitive cancer cell line to the HLDA (IC_{50}: 23 μg/ml) and the ethyl acetate fraction treatment (IC_{50}: 46 μg/ml), suggesting that HLDA and the ethyl acetate fraction exhibit certain degree of selectivity on the growth of melanoma cells, when compared to the other cancer cell types tested in this study.

Little success has emerged over the last 20 years for the treatment of metastatic melanoma because it is often resistant to the current anticancer drugs (Tsao and Soba, 2005). The identification of HLDA as a potent inhibitor of A375 melanoma cells may provide a new candidate for the treatment of melanoma-derived cancers. Thus, the A375 melanoma cell line was chosen for detailed analysis on the anticancer mechanisms of HLDA and the ethyl acetate fraction. Furthermore, their anticancer

activity was also evaluated *in vivo*.

Triterpenes are compounds with a carbon skeleton based on 6 isoprene units and are found widely throughout the plant kingdom. They have relatively complex cyclic structures, most being alcohols, aldehydes, or carboxylic acids. Previous reports indicated that betulinic acid (BA), an example of triterpenes, induced cell cycle arrest at G_1 phase and caused apoptosis in human melanoma B16F10 cells (Sawada *et al.*, 2004). BA also triggered apoptosis in human colorectal carcinoma HCT 116 and prostate carcinoma DU145 by a direct effect on mitochondria followed by caspase-3 activation (Fulda *et al.*, 1998). As shown in this study, HLDA isolated from *Schefflera heptaphylla* (L.) Frodin, formerly known as *Schefflera octophylla* (L.) Harms, was also found to retard the growth of human melanoma A375 cells.

Apoptosis induction is one of the important anticancer therapeutic routes because nearly all anticancer agents kill tumor cells by inducing apoptosis (Ghobrial *et al.*, 2005). Since the mechanisms underlying the process of apoptosis are complex and there is no single discriminative feature, it is necessary to use different methods to determine the mode of cell death. In this study, DNA fragmentation and PARP cleavage, two of the most commonly used indicators of apoptosis, as measured by flow cytometry and Western blotting respectively, were employed to determine the mode of cell death induced by HLDA and the ethyl acetate fraction. Both HLDA and the ethyl acetate

fraction, from which HLDA was derived, have similar properties in inducing apoptosis in the melanoma cells. However, the results from flow cytometry showed that HLDA did not modulate cell cycle of the cancer cells but the ethyl acetate fraction did, suggesting that the growth inhibitory effect of the ethyl acetate fraction was not solely attributed to HLDA but also the other constituents found in the fraction.

Apoptosis is characterized as a well-ordered, programmed sequence of events for cell death, in which loss of mitochondrial membrane potential through the action of the Bcl-2 family of proteins, caspase activation, and DNA fragmentation are the hallmarks (Gilmore *et al.*, 2000). There are two better-characterized pathways of apoptosis, the intrinsic and the extrinsic apoptotic pathways. The upstream initiator caspases, caspase-8 and caspase-9 in the extrinsic and intrinsic pathways respectively, converge to the downstream effector caspases (Weng *et al.*, 2005). The effector caspases then cleave their substrates, such as PARP, so that the cellular, morphological and biochemical alterations of apoptosis are ultimately resulted (Cohen, 1997). In addition to the elevation of apoptotic peak in the DNA histograms of HLDA- and the fraction-treated melanoma cells, PARP cleavage was also observed in the Western blot study. The latter observation not only further consolidates the finding that HLDA and the fraction induced apoptosis in the melanoma cells, it also indicates that caspases might also have been activated to cleave and then inactivate PARP.

Both HLDA and the ethyl acetate fraction from *S. heptaphylla* were shown in this study to retard growth of the human melanoma A375 cells by inducing apoptosis. To further understand the molecular mechanisms of HLDA- and the ethyl acetate fraction-induced apoptosis, two important classes of apoptosis-related proteins, i.e. the Bcl-2 family proteins and caspases were investigated. Results from Western blot analysis showed that the HLDA- and the ethyl acetate fraction-induced apoptosis was associated with depletion of antiapoptotic Bcl-2 family proteins, including Bcl-2 and Bcl-X_L. As the ratio of antiapoptotic to proapoptotic Bcl-2 family proteins is critical to the cellular decision to live or die (Herzig and Christofori, 2002), disturbance of such ratio by HLDA and the fraction in A375 cells could explain for their capability to induce apoptosis. Previous studies have shown that downregulation of Bcl-2 and Bcl-X_L may promote the oligomerization of Bax subfamily proteins, thereby enhancing the release of apoptogenic molecules, such as cytochrome *c*, from mitochondria and thus initiates the degradative cascades of apoptosis (Borner, 2003).

The roles of caspases in the HLDA- and the ethyl acetate fraction-induced apoptosis were studied by using different caspase inhibitors. The results indicated that apoptosis induced by HLDA was solely dependent on caspases, while the fraction-induced apoptosis was executed by both caspase-dependent and caspase-independent events. Apparently, the ethyl acetate fraction contains other

phytochemical(s) that could elicit caspase- independent apoptosis.

Apoptosis can be executed by two main pathways, namely the intrinsic and the extrinsic pathways (Earnshaw *et al.*, 1999). Results from Western blot analysis showed that both of these pathways were activated upon treatment with either HLDA or the ethyl acetate fraction, as indicated by cleavage and thus activation of caspase-8 and caspase-9, which are the hallmark and initiator caspases of the intrinsic and extrinsic pathways, respectively. Preincubation with either caspase-8 or caspase-9 inhibitor could retard the HLDA-induced apoptosis by half in extent. However, the inhibitor retarded the cell death process less prominently in the ethyl acetate fraction-induced apoptosis. This suggests that the apoptosis might be mediated by some caspase-independent events or other initiator caspases, such as caspase-2 and caspase-10.

Bid, a BH3 domain-only protein, which directly induces Bax and Bak oligomerization at the outer mitochondrial membrane, is localized in the cytosolic fraction of cells as an inactive precursor (Grinberg *et al.*, 2002). Cytosolic Bid can be cleaved by caspase-8 or caspase10 in the Fas signaling pathway (Li *et al.*, 1998). Its active form, truncated Bid (tBid), translocates to the mitochondria, induces cytochrome *c* release, and activates the mitochondrial death pathway (Letai *et al.*, 2002) Thus, there is cross-talk mechanism between the extrinsic and the intrinsic apoptotic pathways (Muhlethaler-Mottet *et al.*, 2004).

Interestingly, although both the intrinsic and the extrinsic pathways were activated by HLDA and the ethyl acetate fraction, the presence of an intact Bid protein suggested that the two pathways might be activated independently of one another. However, recent studies suggest that direct degradation of Mcl-1 by TRAIL-activated caspase-8 produces Mcl-1-free Bim that mediates a Bax-dependent apoptotic cascade (Weng *et al.*, 2005; Han *et al.*, 2006). Thus, we cannot rule out the possibility of the presence of unknown candidates that are involved in the cross-talk mechanism between the extrinsic and the intrinsic apoptotic pathways in our system.

By comparing the apoptotic pathways induced by HLDA and the ethyl acetate fraction, it is suggested that the apoptotic effect of the fraction was partially mediated by HLDA, particularly in the initiation of caspase-dependent events and depletion of Bcl-X_L protein. The caspase-independent events, and the depletion of Bcl-2 protein, on the other hand, were attributed to other constituents present in the ethyl acetate fraction.

In this study, the ethyl acetate fraction from *S. heptaphylla* was found active in retarding growth of human melanoma A375 cells, primarily through induction of apoptosis. However, cell-based models cannot accurately model the complex process within animals. Thus, two mouse models, including the DMBA/TPA-induced skin carcinogenesis and the human xenograft model, were chosen to investigate the cancer preventive and therapeutic effects of the fraction *in vivo*, respectively.

The DMBA/TPA-induced skin carcinogenesis model has been widely used to determine the chemopreventive effects of different interventions. For example, the well-known phytochemicals with cancer preventive activities, such as polyphenols from red wine (Soleas *et al.*, 2002), and curcumin from *Curcuma longa* (Limtrakul *et al.*, 1997) had been studied extensively with the DMBA/TPA model.

Initiation induced by a single exposure to the initiator, DMBA, causes mutation in some critical genes (e.g. c-Ha-*ras* gene, a gene involved in epidermal proliferation) in only a few epidermal cells (Yuspa, 1986; Henning *et al.*, 1993). During the promotion stage, repeated application of the promoter, TPA, triggers the epidermal cells to undergo selective clonal expansion due to the acquisition of proliferative advantage, and the ability to evade apoptotic signals (Muto *et al.*, 2006).

The DMBA/TPA-induced skin carcinogenesis model was used in this study to investigate the cancer preventive properties of the ethyl acetate fraction *in vivo*. Significant inhibition of papilloma development was observed in the fraction-treated mice. Moreover, topical application of the fraction at the promotion stage did not cause any lethality, suggesting that the fraction is an effective and safe agent against skin papilloma development by inhibiting TPA-induced promotion.

Betulinic acid, a pentacyclic lupane-type triterpene, is structurally similar to HLDA derived from the ethyl acetate fraction. It is active *in vivo* against

DMBA/TPA-induced papilloma (Yasukawa *et al.*, 1991). Surprisingly, dose of the ethyl acetate fraction as low as 4 mg/ml (i.e. 0.8 mg ethyl acetate fraction dissolved in 200 µl acetone was applied to a 25 g mouse in each treatment), was shown to inhibit the papilloma development significantly in the DMBA/TPA model, which is more potent than that of betulinic acid (effective dose as 5 µmol, i.e. 2.28 mg in each treatment) (Yasukawa *et al.*, 1991).

Human tumor xenografts implanted subcutaneously (s.c.) into immunosuppressed mice have played a significant role in preclinical anticancer drug development in the past few years (Bae *et al.*, 2005). Melanoma A375 cells, which were most responsive to the ethyl acetate fraction in the *in vitro* studies, were implanted into nude mice for studying the cancer therapeutic effect of the ethyl acetate fraction *in vivo*. We observed that the A375 xenografts were significantly reduced in the fraction-treated groups when compared to the control group, in term of tumor weight.

As reported by Pisha *et al.* (1995), betulinic acid exhibited no toxic effect in mice implanted with human xenograft at a concentration of 500 mg/kg, while a dose of 5 mg/kg was shown to regress xenograft size significantly. In this study, the ethyl acetate fraction regressed the size of A375 xenograft significantly at a dose of 2 mg/ml (i.e. 16 mg/kg) but no toxic effect at 320 mg/kg. Apparently, the data showed that the ethyl acetate fraction served better as a chemopreventive agent than a chemotherapeutic

agent since 40 mg/ml of the fraction could completely inhibit the papilloma incidence in the multistage skin carcinogenesis model whereas the weight of A375 xenografts in athymic mice model were only retarded by 84%. The reasons for these observed discrepancies between the two models might be due to the differences in their principles and duration of treatment. In general, an experimental drug is considered to be active when the percentage of growth inhibition of mouse tumor is at least 58% (Mattern *et al.*, 1988). Thus, results from *in vivo* studies strongly evidenced that the ethyl acetate fraction is an active anticancer agent.

This is the first report showing that the components from *S. heptaphylla* possesses strong inhibitory activities against melanoma growth *in vivo* and *in vitro*. This suggests potential therapeutic roles of the ethyl acetate fraction as an adjuvant for the treatment of melanoma (Bae *et al.*, 2005). However, an in-depth study to define the active agent(s) in the ethyl acetate fraction capable of affording the observed antitumor-promoting effect is highly warranted.

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